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Coupled In Vitro Import of U snRNPs and SMN, the Spinal Muscular Atrophy Protein

Usha Narayanan,[1] Tilmann Achsel,[2,3] Reinhard Lührmann,[2] and A. Gregory Matera[1,*]
[1]Department of Genetics
Case Western Reserve University School of Medicine
Cleveland, Ohio 44106
[2]Max Planck Institut für Biophysikalische Chemie
Abteilung Zellulare Biochemie
D-37070 Göttingen
Germany

Summary

Cytoplasmic assembly of Sm-class small nuclear ribonucleoproteins (snRNPs) is a central process in eukaryotic gene expression. A large macromolecular complex containing the survival of motor neurons (SMN) protein is required for proper snRNP assembly in vivo. Defects in SMN function lead to a human neuromuscular disorder, spinal muscular atrophy (SMA). SMN protein localizes to both nuclear and cytoplasmic compartments, and a reduction in nuclear levels of SMN is correlated with the disease. The mechanism of SMN nuclear import, however, is unknown. Using digitonin-permeabilized cells, we show that SMN import depends on the presence of Sm snRNPs. Conversely, import of labeled U1 snRNPs was SMN complex dependent. Thus, import of SMN and U snRNPs are coupled in vitro. Furthermore, we identify nuclear import defects in SMA patient-derived SMN mutants, uncovering a potential mechanism for SMN dysfunction.

Introduction

Spinal muscular atrophy (SMA) is a common genetic disorder caused by homozygous deletion or loss-of-function mutations in the *survival of motor neurons 1* gene, *SMN1* (Lefebvre et al., 1995). Disease pathology is characterized by diminished production of SMN protein, resulting in motor neuron degeneration in the anterior horn of the spinal cord, followed by muscular atrophy and paralysis (reviewed in Frugier et al., 2002; Talbot and Davies, 2001; Wirth, 2000). Whereas phenotypic severity is inversely proportional to the overall SMN expression level, the molecular etiology of the disease is unknown.

Current efforts aimed at understanding the molecular defects underlying these loss-of-function mutations fall into two major categories. One hypothesis is that SMN has a motor neuron-specific function, and that loss of this specialized activity results in SMA. Accordingly, SMN has been localized to axonal processes and neuromuscular junctions (Bechade et al., 1999; Pagliardini et al., 2000; Cifuentes-Diaz et al., 2002; Fan and Simard, 2002; Rossoll et al., 2002; Chan et al., 2003); however, mechanistic studies have yet to emerge. The second theory holds that SMN performs a general cellular function, and that perturbation of this activity has particularly dramatic effects on motor neurons. Consistent with this idea, SMN protein is ubiquitously expressed and is required for assembly of uridine-rich small nuclear ribonucleoproteins (U snRNPs) in vivo (Meister et al., 2001; Meister and Fischer, 2002; Pellizzoni et al., 2002). Thus, genetic and biochemical studies have established an essential cellular function for SMN. Despite this progress, it is clear that the full complement of roles played by SMN have yet to be elucidated.

SMN forms large macromolecular complexes with other core components, collectively called Gemins (reviewed in Meister et al., 2002; Paushkin et al., 2002). While these factors are distributed throughout the cytoplasm, they accumulate in discrete nuclear foci called Cajal bodies (Ogg and Lamond, 2002; Gall, 2003; Tucker and Matera, 2004). Nuclear functions for the SMN complex have not been described, but loss of SMN nuclear foci correlates with the disease phenotype (Coovert et al., 1997; Lefebvre et al., 1997).

Assembly of Sm-class snRNPs is a stepwise process that takes place in multiple subcellular compartments (Will and Lührmann, 2001; Jady et al., 2003). SMN associates with snRNPs throughout the cytoplasmic phase of this pathway, including recognition of the snRNA export complex, assembly of the Sm core, hypermethylation of the RNA 5′ cap, and trimming of its 3′ end (Fischer et al., 1997; Massenet et al., 2002; Mouaikel et al., 2003). Further, SMN is known to form a cytoplasmic, preimport RNP complex with the import adaptor, snurportin1 (Narayanan et al., 2002). However, as a functional role for this complex has yet to be established, we developed an in vitro system to address the mechanism of SMN nuclear import. Taken together, our results identify an additional role for the SMN complex in U snRNP maturation and import.

Results

GFP-SMN Functions as an Efficient Import Substrate

The digitonin-permeabilized HeLa cell system is a well-established method to study nuclear transport (Adam et al., 1992; Dingwall and Palacios, 1998). For use as import substrates, we GFP tagged various SMN constructs. The proteins were synthesized in rabbit reticulocyte lysate using the coupled transcription-translation system. Western blot analyes of the programmed lysates identified a single band of the expected size in each lane (data not shown). Using digitonin-permeabilized cells, we demonstrated that GFP-SMN import is active, temperature dependent, and mediated by cytosolic factors (Figure 1 and data not shown). GFP-SMN was predominantly nuclear after incubation for 60 min at 30°C (Figure 1A), whereas it was cytoplasmic following incubation for the same time period at 4°C (Figure 1A).

*Correspondence: a.matera@case.edu
[3]Present address: IRCCS Fondazione Santa Lucia Neurobiologia, Via Ardeatina 306, 00179 Rome, Italy.

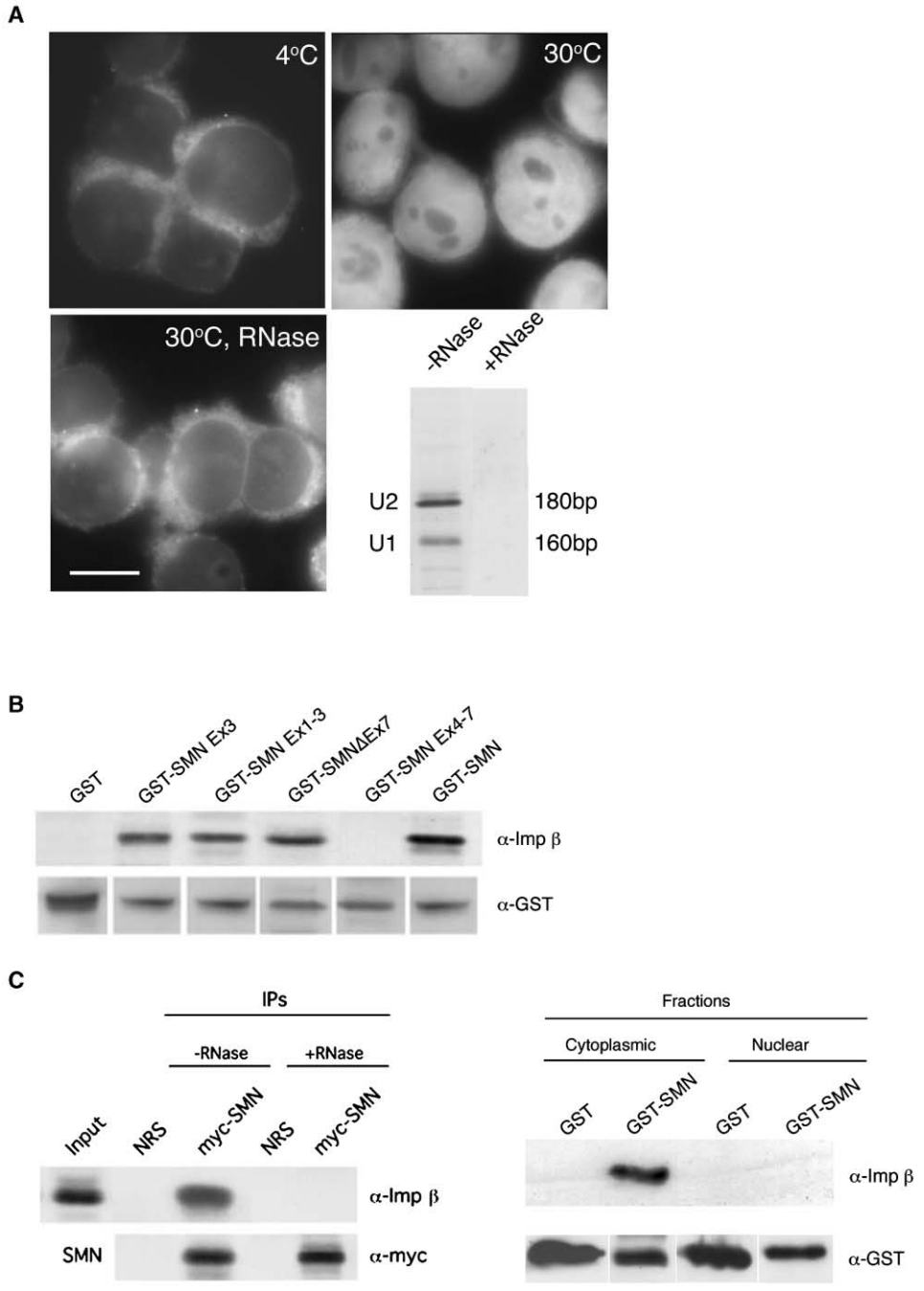

Figure 1. RNA Is Essential for SMN Import

(A) Import assays were conducted in vitro by permeabilizing HeLa cells with digitonin and incubating them for 1 hr at 4°C (negative control) or 30°C (positive control). Transport reactions were performed with GFP-SMN generated in untreated or RNase-treated rabbit reticulocyte lysate. On RNase treatment, the RNA levels were monitored by Northern blotting. Bar, 10 μm.

(B) The SMN domain encoded by exon3 is both necessary and sufficient for importin β binding in vitro. GST pull-downs were set up with a panel of SMN constructs and recombinant importin β; GST alone served as a negative control. The pull-downs were analyzed by Western blotting for importin β and GST (loading control).

(C) RNA stabilizes the SMN-Importin β interaction in vivo (left). HeLa cells were transiently transfected with myc-SMN; untransfected cells served as a negative control. Immunoprecipitations (IPs) were performed from total HeLa cell lysates with monoclonal antibodies against myc either in the presence or absence of RNase. The IPs were analyzed by Western blotting with α-importin β; α-myc was used as a loading control. SMN and importin β interact in the cytoplasm (right). HeLa cells were fractionated and pull-downs were set up against the nuclear and cytoplasmic pools using GST-SMN or GST alone (negative control). The results were assessed by Western blotting with α-importin β and α-GST (loading control).

RNA Is Essential for SMN Import

Having defined the temporal parameters for nuclear transport, we determined whether SMN import was RNA dependent. Following translation of GFP-SMN, the reticulocyte lysate was incubated in the presence or absence of RNases A and T1 prior to use in the transport assay. Northern blotting was used to monitor RNA integrity in the lysate (Figure 1A). RNase-treated lysate did not sustain import, whereas untreated or mock-treated lysate efficiently imported GFP-SMN (Figure 1A). Thus, nuclear import of GFP-SMN requires RNA in the reconstituted cytosol.

Previously, we showed that GST-SMN binds recombinant importin β in vitro, potentially serving as its putative import receptor (Narayanan et al., 2002). Upon mapping the interaction using various GST-SMN subfragments, we found that the importin β interaction domain was contained within sequences encoded by SMN exon 3 (Figure 1B). Intriguingly, this region of SMN is also known to bind Sm proteins (Bühler et al., 1999). The direct interaction of SMN and importin β seemed at odds with a requirement for RNA in SMN import. As GST-pull-down experiments are highly sensitive, we hypothesized that, in vivo, RNA might be required in order to stabilize the interaction. To test this idea, we transfected HeLa cells with myc-tagged SMN and immunoprecipitated with anti-myc antibodies using cell lysates that were either treated or untreated with ribonuclease. We then assayed for coprecipitation of importin β by Western blotting. Importin β was recovered only in the absence of RNase (Figure 1C, left panels), and in a separate GST-pull-down experiment, complexes between SMN and importin β were only detected in the cytoplasm (Figure 1C, right). Thus, the interaction of SMN and importin β in vivo is cytoplasmic and RNA mediated.

SMN Import Requires snRNPs

As SMN binds to the C-terminal tails of Sm proteins (Friesen and Dreyfuss, 2000; Brahms et al., 2001) and forms cytoplasmic complexes with snurportin1 in vivo (Narayanan et al., 2002; Massenet et al., 2002), we hypothesized that U snRNPs are the requisite RNAs for GFP-SMN import (Figure 1). To test this, we immunodepleted snRNPs from the reticulocyte lysate using anti-Sm antibodies and synthesized GFP-SMN in the snRNP-depleted lysate for use in the transport assay. Loss of snRNPs from the lysate was monitored by Western blotting (Figure 2A). Control lysates were depleted with antibodies against U2B″, a U2 snRNP-specific protein that is imported independently, binding the RNP only in the nucleus (Hetzer and Mattaj, 2000). Significantly, depletion of snRNPs but not U2B″ inhibited GFP-SMN import (Figure 2B). To establish that the effect was due to the snRNPs present in the reticulocyte lysate, we added purified snRNPs (Bach et al., 1990) to the depleted lysates and performed the import assay. As shown, adding either U1 or U2 snRNPs to the snRNP-depleted lysate rescued GFP-SMN import (Figure 2B). As the purified snRNP fractions lacked snurportin and importin β (Huber et al. 2002 and data not shown), we conclude that SMN nuclear import is dependent upon the presence of U snRNPs.

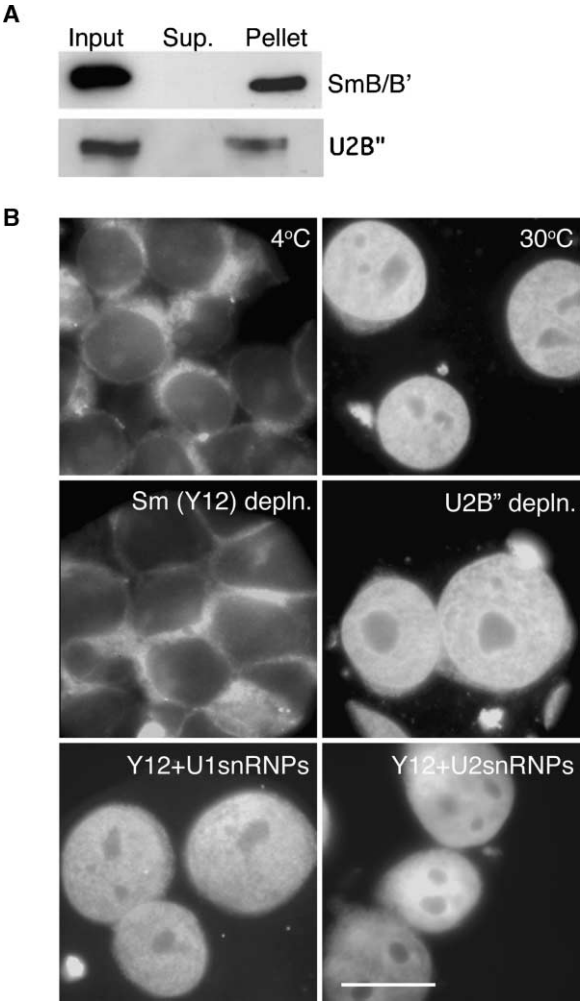

Figure 2. SMN Import Requires snRNPs

(A) Reticulocyte lysates used to supplement import reactions were immunodepleted with α-Sm (mAb Y12) or α-U2B″ (mAb 4G3) antibodies and subsequently used to synthesize GFP-SMN. As a control, part of the pellet and supernatant from the depleted lysates were run on a polyacrylamide gel and analyzed by Western blotting. Input lanes represent 12% of the total.

(B) GFP-SMN import was monitored in the presence of reticulocyte lysate and an energy regenerating system at 4°C or 30°C (top panels). In the middle panels, depleted lysates were used to study import. In the bottom panels, SMN import defects caused by snRNP depletion were rescued using 0.2 μM purified, unlabeled U1 or U2 snRNPs. Bar, 10 μm.

Importin β Is Required for SMN Import

Previous studies have shown that nuclear transport of U snRNPs depends upon importin β (Palacios et al. 1997). If SMN import depends on snRNPs, then it follows that importin β should mediate SMN transport. Therefore, we depleted importin β from the reticulocyte lysate using a GST fusion of the importin β binding (IBB) domain of importin α (Görlich et al., 1996). GST alone was used as a control. Western blotting of the depleted lysate confirmed removal of importin β (Figure 3B). GFP-SMN was translated in the depleted lysates for use in the import assay. As shown in Figure 3A, GFP-SMN import was disrupted by removal of importin β and was unaf-

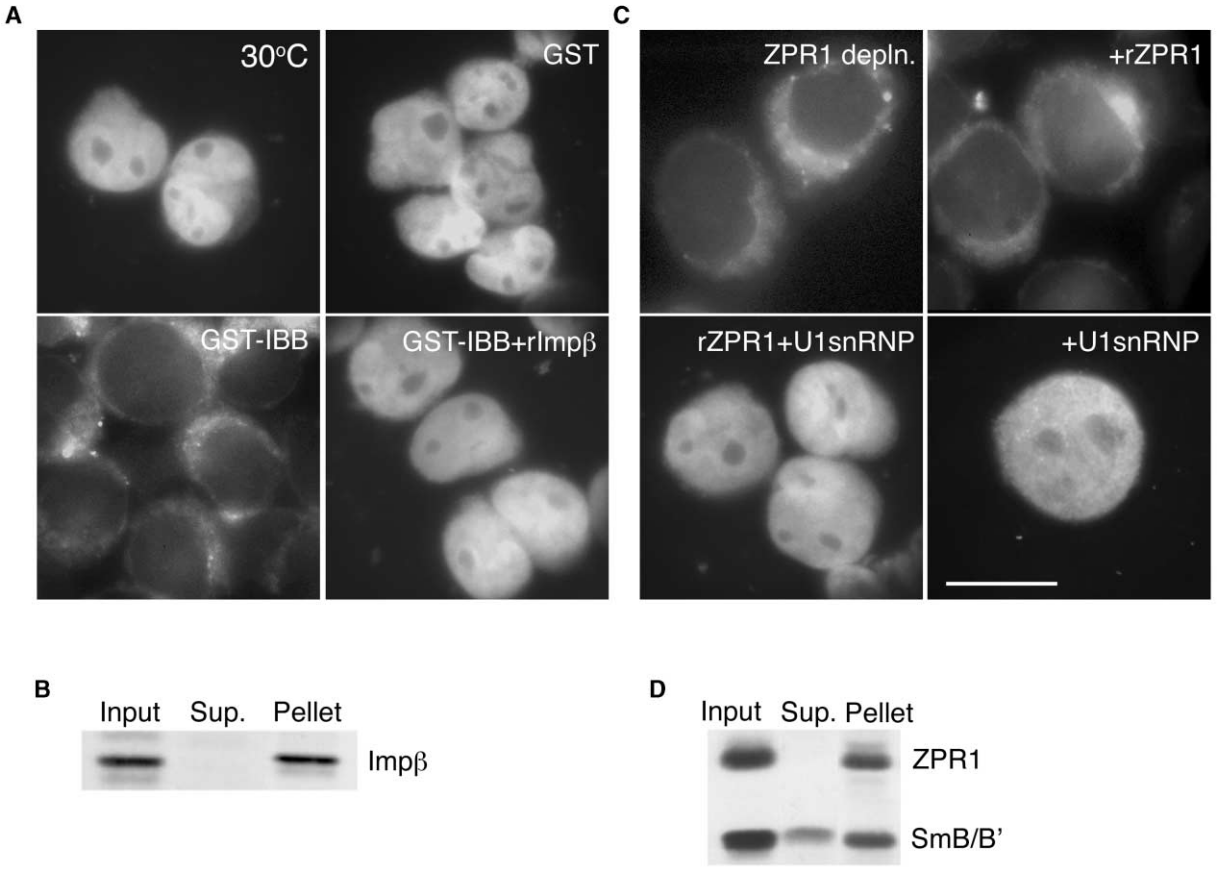

Figure 3. Importin β Is the SMN Import Receptor, whereas ZPR1 Does Not Play a Direct Role in SMN Import

(A) GFP-SMN import was assayed in the presence or absence of importin β. Depletions were conducted prior to GFP-SMN synthesis using the importin β binding domain of importin α fused to GST (GST-IBB); GST alone served as a control. GFP-SMN import was rescued using 100 ng of recombinant importin β (GST-IBB + rImpβ).

(B) The depletions were confirmed by Western blotting. Input lanes represent 12% of the total.

(C) ZPR1 was immunodepleted from the reticulocyte lysates and GFP-SMN import was analyzed. Defective import in the ZPR1-depleted lysates was restored with 3 μg rZPR1, 3 μg rZPR1 + 0.2 μM U1 snRNPs, or 0.2 μM U1 snRNPs alone, as indicated. Bar, 10 μm.

(D) The immunodepletions were monitored by Western blotting with α-ZPR1 and α-SmB/B′ (Y12) antibodies. Input lanes represent 12% of the total.

fected by depletion with GST alone. Strikingly, adding recombinant importin β rescued GFP-SMN import (Figure 3A). These data demonstrate that importin β indeed serves as the cognate import receptor for SMN.

ZPR1 Forms Complexes with snRNPs and SMN but Is Not Essential for Import

A signaling protein, called ZPR1, has been implicated in localization of SMN to the nucleus. The interaction of SMN and ZPR1 is thought to be indirect, mediated by unknown factors (Gangwani et al., 2001). Both proteins redistribute from the cytoplasm to the nucleus in response to serum mitogens, requiring their respective C-terminal domains for the process (Gangwani et al., 2001). The data outline a potential signaling pathway connecting cellular proliferation factors with the snRNP biogenesis machinery (Matera and Hebert, 2001). However, whether the requirement for ZPR1 was upstream, downstream, or directly in the nuclear transport event of SMN is unknown (Gangwani et al., 2001).

To assess if ZPR1 is essential for SMN import in vitro, we performed the import assay using reticulocyte lysate depleted for ZPR1. As shown in Figure 3C, depletion with anti-ZPR1 abrogated GFP-SMN import, but adding back recombinant ZPR1 alone did not restore the defect. Curiously, we found that depleting ZPR1 also cleared a significant fraction of snRNP proteins from the lysate (Figure 3D). Accordingly, adding ZPR1 and purified U1 snRNPs to the depleted lysates rescued SMN import (Figure 3C). However, ZPR1 is not strictly required for SMN import, as adding U1 snRNPs alone proved sufficient for rescue (Figure 3C). These experiments reveal that although ZPR1 forms cytoplasmic complexes with SMN and U snRNPs, it is not crucial for SMN import.

SMN Complex Is Required for U snRNP Import

In somatic cells, nuclear localization of Sm-class snRNPs involves a bipartite signal. One part of the signal is comprised of the trimethylguanosine (TMG) cap and the other by an unkown factor that binds the Sm-core domain (Fischer et al., 1993; Marshallsay and Lührmann, 1994; Huber et al., 1998). These distinct pathways (Huber

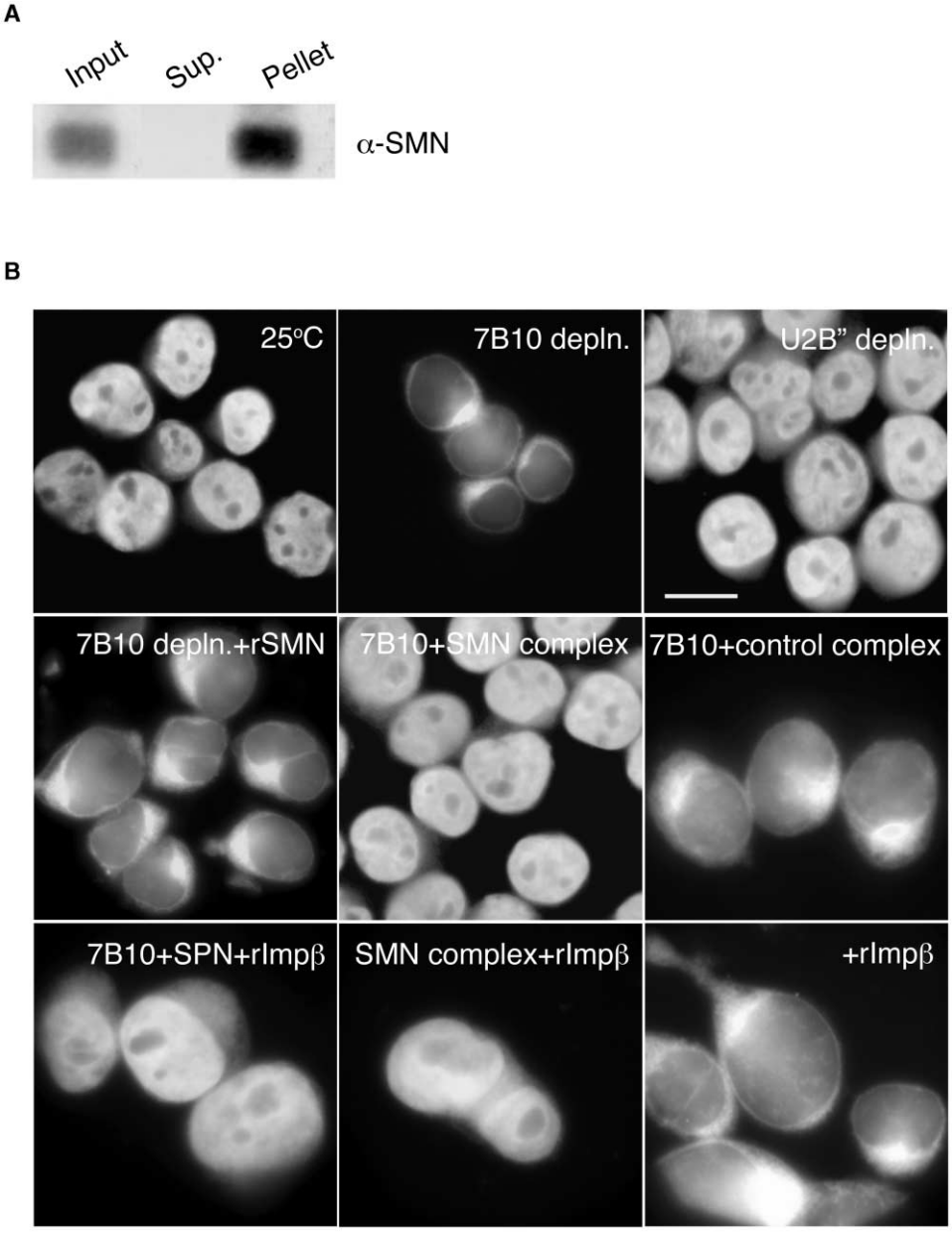

Figure 4. SMN Complex Is Required for snRNP Import

Cy3-labeled U1 snRNPs were used as import substrates in digitonin-permeabilized HeLa cells. The cytosol was reconstituted using rabbit reticulocyte lysate. The in vitro import assays were conducted for 30 min at 25°C. Import was examined using SMN- or U2B″-depleted lysates (mAbs 7B10 and 4G3, respectively); the depletions were confirmed by Western blot analyses. Two micrograms of recombinant SMN (rSMN) was added to the reaction to attempt rescue of defective import. SPN and importin β were added in 5-fold molar excess over U1 snRNPs to the SMN-depleted lysate to restore U1 import. One hundred nanograms of purified SMN complex or a control fraction was added to rescue Cy3-U1 snRNP import defects seen upon SMN depletion. In two of the panels, import assays were conducted without added reticulocyte lysate, using recombinant importin β, Cy3-U1 snRNPs, and with or without purified SMN complexes (400 ng), His-Ran, GTP, and an ATP regenerating system. Bar, 10 μm.

et al., 2002) converge upon importin β for passing through nuclear pores (Palacios et al., 1997). As snRNP and SMN import appear to be linked (Figures 2 and 3), it follows that if removal of snRNPs from the cytosol abolishes SMN import, then SMN depletion should impair snRNP import. Therefore, we assayed the import of Cy3-labeled U1 snRNPs, supplemented with control or SMN-depleted reticulocyte lysates. Figure 4 reveals that SMN depletion inhibits import of U1 snRNPs, whereas mock-depleted or anti-U2B″-depleted lysates fully support transport. Notably, supplementing SMN-depleted lysates with recombinant SMN protein failed to rescue U1 import, indicating the requirement for additional factors. Since anti-SMN antibodies pull down a number of additional proteins, we hypothesized that the SMN complex might contain these putative accessory import factors.

To this end, we added purified, functional SMN complexes (the kind gift of G. Dreyfuss and J. Yong; for details see Pellizzoni et al. 2002) to the SMN-depleted lysate and found that they successfully rescued U1 import (Figure 4B); control fractions of nonspecific proteins failed in this regard (Figure 4B). Western blot analyses confirmed the absence of importin β or snurportin in the purified SMN complexes (see Supplemental Data). Thus the SMN complex, or a subset thereof, is sufficient to rescue U1 import in SMN-depleted lysate.

Given that U snRNP import can proceed by either of two independent pathways (Fischer et al. 1993; Marshallsay and Lührmann 1994; Huber et al. 2002), we reasoned that the SMN complex could, in principle, be *sufficient* for rescue of U1 import but not *necessary*. In fact, Huber et al. (2002) showed that a 5-fold molar excess of recombinant snurportin and importin β was sufficient for in vitro import of U1 snRNPs. Consistent with the existence of the two pathways, we found that U1 snRNPs in the SMN-depleted lysate could be chased into the nucleus by adding a 5-fold excess of snurportin and importin β (Figure 4B). The fact that Cy3-U1 import could be rescued from SMN-depleted lysates by adding importin β with *either* snurportin *or* SMN complex suggested that snRNP import could proceed in the absence of exogenous snurportin. We therefore assayed Cy3-U1 import using cytosol entirely reconstituted with purified components (i.e., without reticulocyte lysate). To ensure the removal of endogenous snurportin, we preincubated the digitonin permeabilized cells in transport buffer for 10 min prior to performing the assay (Huber et al. 2002). As shown in Figure 4B, adding purified SMN complexes, GTP, an energy regenerating system, along with recombinant Ran and importin β was sufficient for U1 snRNP import. When the purified SMN complexes were omitted from the mixture, these factors alone were insufficient for import of Cy3-U1 snRNPs (Figure 4B).

SMN and U snRNP Import Are Linked

The rate of U snRNP import in somatic cells is greatly accelerated by 5′ cap hypermethylation (Fischer et al., 1994). Thus, the kinetics of GFP-SMN import should be slowed by factors that interfere with U1 import. To that end, we assayed U snRNP and SMN import in parallel for 60 min at 25°C in the presence of increasing amounts of either 7-methylguanosine (m7G) or TMG cap analogs. Figure 5A reveals that high concentrations of TMG caps inhibited import of Cy3-U1 and GFP-SMN, whereas equivalent amounts of m7G caps had no effect. Thus, factors that interfere with snurportin can affect the efficiency of both U1 and SMN import (Figure 5A). Likewise, depletion of SMN complex interfered with U1 import (Figure 4). Collectively, these experiments suggest that an excess of SMN complex might accelerate cap-independent U1 import. We therefore assessed Cy3-U1 import in the presence of 0.2 μM TMG caps with lysate supplemented with 400 ng of purified SMN complexes. As shown in Figure 5A, we found that, just as adding an excess of snurportin and importin β chased snRNPs into the nucleus, an excess of SMN complex could also overcome the TMG cap inhibition.

In establishing the temporal framework for studying SMN and U snRNP import, we found that Cy3-U1 could be detected in the nucleus after 30 min, whereas appreciable GFP-SMN signal was visible only after 60 min. In attempting to reconcile this apparent discrepancy, we investigated whether snRNPs might be rate limiting in the reticulocyte lysate. As shown in Figure 5B, we found that adding purified U1 snRNPs to the lysate accelerated the kinetics of GFP-SMN import. Upon addition of 0.1 μM and 0.2 μM snRNPs, nuclear GFP-SMN was detected after 40 and 30 min, respectively (Figure 5B and data not shown). Thus, the addition of U1 correlated with the time needed for GFP-SMN import. To directly visualize coupled import of snRNPs and SMN at 30 min with 0.2μM snRNPs, we studied import using both Cy3-U1 and GFP-SMN as import substrates in the same reaction. We found that both U1 and SMN were imported into the nucleus at 30 min in the presence of added snRNPs at 30°C, but neither was imported at 4°C (Figure 5B and data not shown). Together, these results demonstrate the interdependence of U snRNP and SMN import, implicating a pivotal role for the SMN complex in the cap-independent snRNP import pathway.

SMN Mutations Disrupt Import

Having established the link between SMN and U snRNP import, we wanted to identify SMN protein motifs necessary for import and to further test the activities of biologically relevant mutations. A cartoon detailing the domain structure of the SMN protein is shown in Figure 6A. The most common SMA mutation produces a truncated SMN isoform lacking the C-terminal 15 amino acids corresponding to the region encoded by exon 7 (reviewed in Frugier et al., 2002). This isoform, SMNΔEx7, is relatively unstable (Lorson and Androphy, 2000) and displays slightly reduced in vitro binding affinities to Sm proteins (Pellizzoni et al., 1999; Hebert et al., 2001) and the snRNA cap hypermethylase, Tgs1 (Mouaikel et al., 2003). Curiously, the GFP-SMNΔEx7 mutant was successfully imported (Figure 6B) and bound efficiently to importin β in a GST pull-down (Figure 1B). Just as with the full-length protein, SMNΔEx7 import was inhibited by snRNP depletion and restored by addition of purified snRNPs (Figure 6B). Thus, exon 7 sequences do not appear to contribute to SMN import in digitonin-permeabilized HeLa cells.

Two important motifs within SMN are the Tudor domain, encompassed by sequences in exon 3, and the Y-G box, encoded by exon 6. The Tudor domain is primarily responsible for binding methylated Sm proteins (Bühler et al., 1999; Hebert et al., 2001; Brahms et al., 2001), whereas the Y-G-rich motif contains a modular self-oligomerization activity (Lorson et al., 1998). We created two internal deletions spanning these domains within GFP-SMN and tested their import efficiencies. Strikingly, the SMNΔEx3 and SMNΔEx6 mutations strongly inhibited import (Figure 6B). Since deletion of the Tudor domain (ΔEx3) produces a protein incapable of binding snRNPs, this finding is completely consistent with the results described above. However, disruption of SMN import by the Y-G box (ΔEx6) deletion is noteworthy, as it suggests the presence of oligomeric SMN in the active import complex (see Discussion). We also created two GFP-SMN constructs bearing missense mutations derived from patients with the severe form of

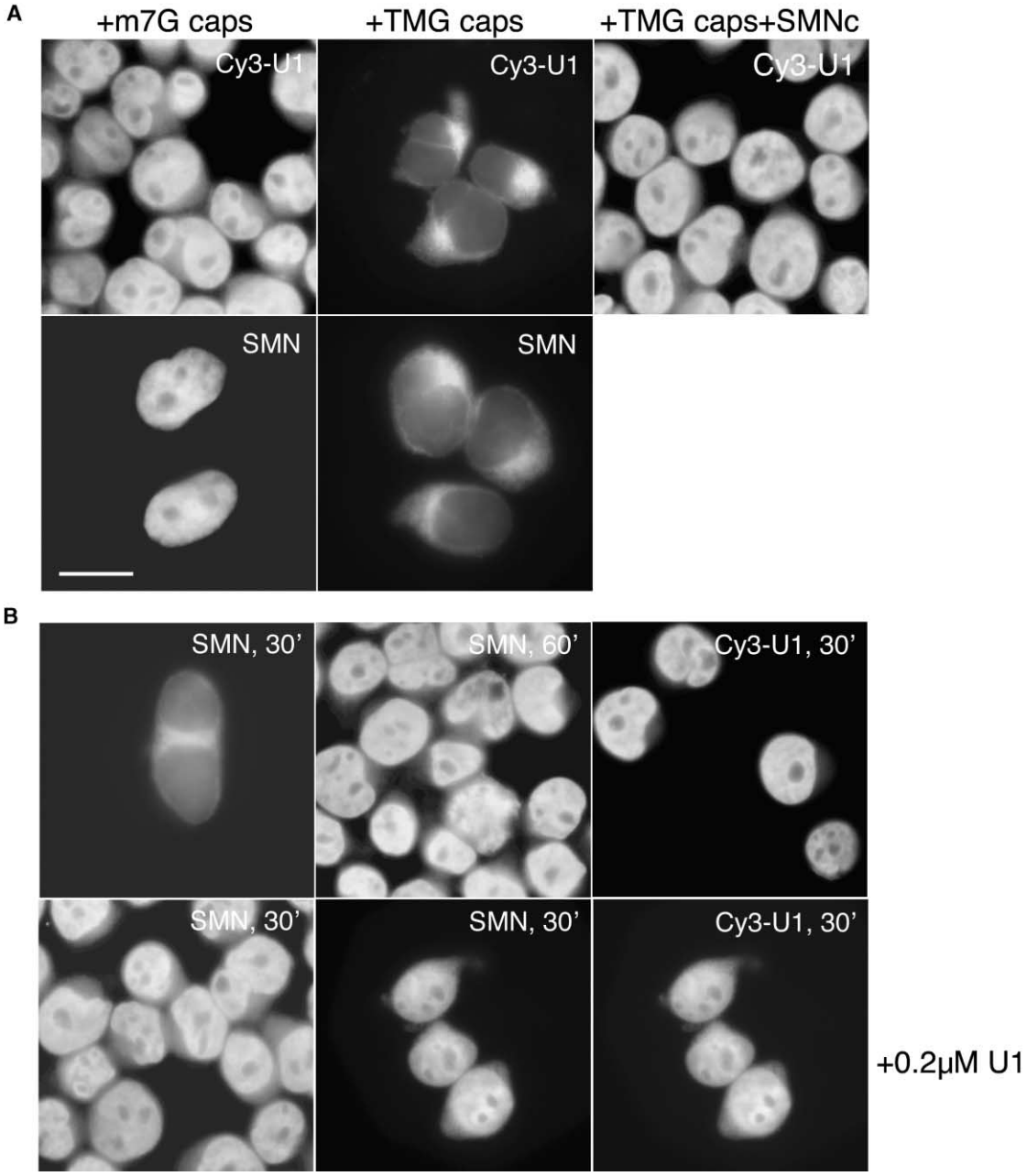

Figure 5. SMN and U snRNP Import Are Coupled

(A) Cy3-U1 import kinetics can be accelerated by supplementing the lysate with purified SMN complexes. Cy3-U1 snRNP and GFP-SMN import were assayed at 25°C using the digitonin-permeabilized HeLa cell assay in the presence of 20 μM TMG caps (with or without 200 ng purified SMN complexes). U1 and SMN import was also assayed with 20 μM m7G caps as a control.

(B) Addition of purified U snRNPs accelerates GFP-SMN import. GFP-SMN or Cy3-U1 snRNP import assays were conducted in digitonin-permeabilized HeLa cells at 25°C for either 30 min or 60 min. GFP-SMN import was also examined at 25°C for 30 min in the presence of 0.2 μM purified U1 snRNPs (bottom panels). Bar, 10 μm.

SMA. Interestingly, both the E134K and Y272C constructs were completely defective for transport (Figure 6B). The E134K mutation maps within the Tudor domain and is quite rare, whereas the Y272C mutation falls within the Y-G box oligomerization motif, the region most commonly targeted by SMA missense mutations (Wirth, 2000).

In light of the import assay results, we tested the various SMN constructs for their abilities to interact with importin β and found that the binding activity mapped to the Tudor domain (Figures 1B and 6C). This result was surprising because the Tudor domain was shown to bind Sm proteins, and the E134K mutation is thought to interfere with this interaction (Bühler et al., 1999; Selenko et al., 2001; Sprangers et al., 2003). However, the Sm binding activity of this mutant had previously been

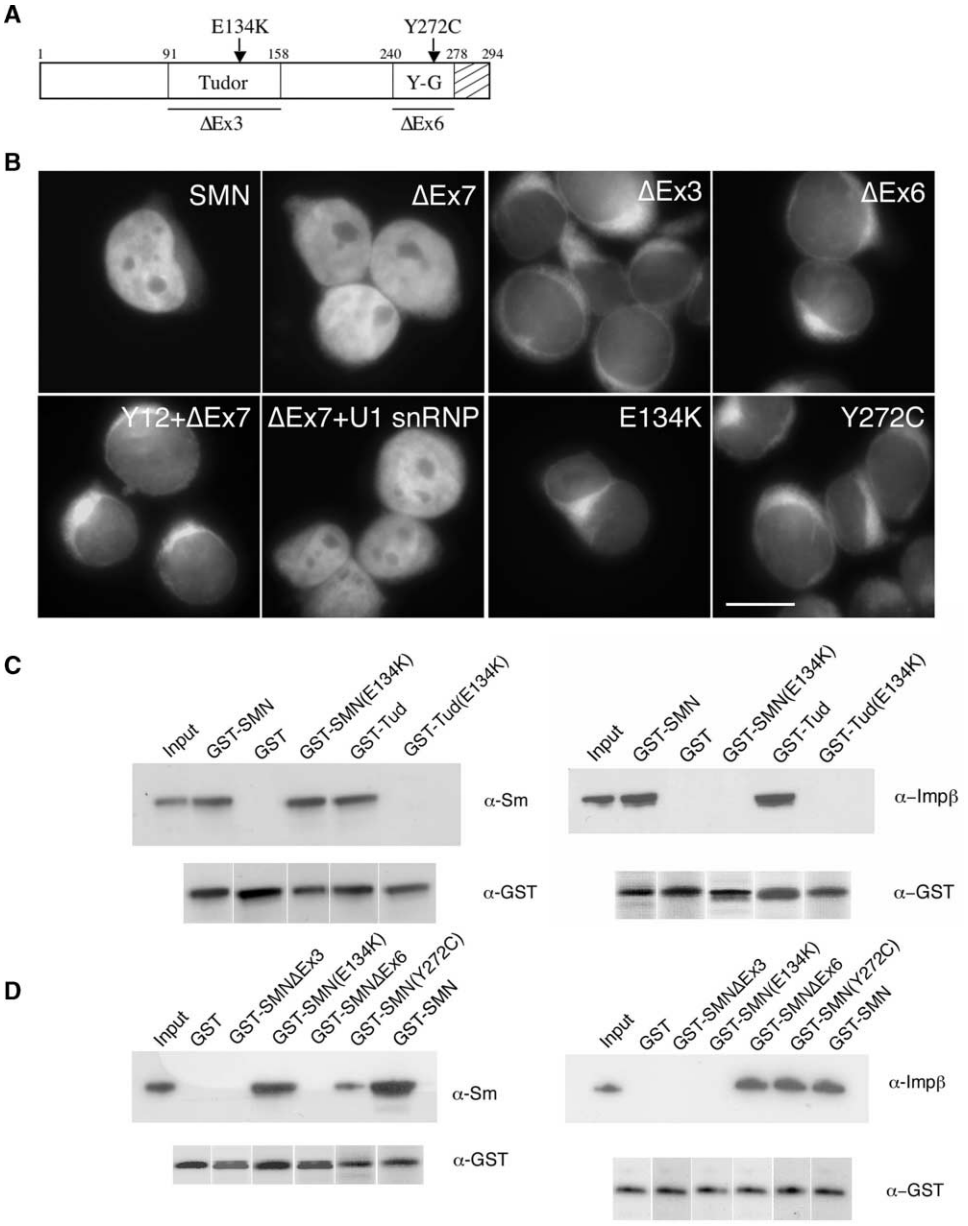

Figure 6. SMN Mutations Defective for Sm Binding or Self-Oligomerization Disrupt SMN Import

(A) Schematic of SMN, exhibiting functional domains. The Tudor domain and Y-G box motif are indicated; lines represent the internal deletions (ΔEx3, ΔEx6), asterisks the point mutations (E134K, Y272C), and the shaded box depicts the C-terminal deletion (ΔEx7).

(B) Digitonin-permeabilized HeLa cells were used for in vitro import assays with the indicated GFP-SMN mutants for 60 min at 30°C. In the indicated panel, reticulocyte lysate was immunodepleted with α-Sm antibodies (mAb Y12). GFP-SMNΔEx7 import was then assayed without (left) or with (right) addition of purified U1 snRNPs (0.2 μM). Bar, 10 μm.

(C) GST pull-down assays were carried out with full-length GST-SMN(wt or E134K) or the Tudor domain of SMN, GST-Tud(wt or E134K) and recombinant SmB′ or importin β as indicated; GST alone served as a negative control. Western blot analyses were carried out, and the blots were probed with α-SmB′ or α-importin β; α-GST was used as a loading control.

(D) GST pull-down assays were conducted with wild-type and the following GST-tagged SMN mutants: E134K, ΔEx3, ΔEx6, and Y272C in the presence of SmB/B′ or importin β as indicated; GST alone served as a specificity control. The pull-downs were analyzed by Western blotting and probed with α-Sm or α-importin β as indicated; α-GST was used as a loading control.

studied in the context of a Tudor domain subconstruct and not the full-length protein (Selenko et al., 2001; Jones et al. 2001). Therefore, we found the E134K allele particularly interesting, as it showed pronounced import defects (Figure 6B). Hence, we created a full-length version of GST-SMN(E134K) and compared its Sm binding capacity to that of the equivalent Tudor domain construct, GST-Tud(E134K). Remarkably, we found that GST-SMN(E134K) and the wild-type construct bound recombinant SmB′ with similar affinities. However, the mutant was completely incapable of binding importin β (Figure 6C). Consistent with previous results (Selenko

et al. 2001; Jones et al. 2001), we confirmed that GST-Tud(E134K) failed to bind SmB′ (Figure 6C). Conclusively, importin β failed to bind E134K, both in the context of the Tudor domain and the full-length protein (Figure 6C). These results show that SMN(E134K) can bind to Sm proteins but not to importin β, suggesting a potential mechanism for SMA disease pathogenesis (see Discussion).

We also compared the other mutants in our study for differential Sm and importin β binding. Whereas the Y-G box deletion (ΔEx6) was unable to bind to Sm proteins, it was fully functional for interaction with importin β (Figure 6D). Moreover, the Y272C mutant displayed reduced binding to Sm proteins but maintained binding to importin β (Figure 6D), reaffirming a role for Sm binding defects at the cellular level in SMA. As expected, the Tudor domain deletion (ΔEx3) failed to bind both Sm proteins and importin β (Figure 6D). In summary, the mutational analyses establish the roles of two important activities in efficient SMN import: Sm protein binding and self-oligomerization.

Discussion

The SMN complex is fast emerging as the common denominator in the biogenesis of many small RNPs (Meister et al., 2002; Paushkin et al., 2002; Terns and Terns, 2001). Current theories hold that SMN functions as a quality-control or specificity factor at multiple stages of cytoplasmic RNP assembly (Massenet et al., 2002; Meister and Fischer, 2002; Mouaikel et al., 2003; Narayanan et al., 2002; Pellizzoni et al., 2002). Our results demonstrate that SMN and snRNP import are interdependent, indicating that SMN is complexed with snRNPs much longer than previously envisioned. The coupling of U snRNP and SMN import opens possibilities for extending SMN's putative chaperoning activity beyond the primary cytoplasmic assembly steps and well into the nucleus.

Recently, nuclear substructures called Cajal bodies (CBs) were proposed to be sites of secondary RNP maturation (Jady et al., 2003); previous studies also infer the existence of a nuclear RNP assembly pathway involving CBs (Sleeman and Lamond, 1999; Sleeman et al., 2003). We therefore anticipated that newly imported GFP-SMN would be first detected in CBs. However, our experiments did not reveal focal accumulations of GFP-SMN. It seems likely that the vast excess of in vitro translated GFP-SMN in these experiments overwhelms the limited number of CB binding sites. However, the kinetics of GFP-SMN import we observed are completely consistent with those of FP-tagged Sm proteins. Microinjection experiments (Sleeman and Lamond, 1999) showed that GFP-SmB appeared diffusely throughout the nucleoplasm at the earliest time points (1 hr post-injection). At later time points (2–4 hr), FP-Sm proteins accumulated primarily in CBs before proceeding on to speckles (6–15 hr). Thus, the earliest post-import pattern observed for the core snRNP proteins agrees with the kinetics of GFP-SMN import studied here.

SMN Import Factors

Prior to import, SMN can be detected in a complex with U snRNPs and the import factors snurportin and importin β (Narayanan et al., 2002). In this study, we found that depleting importin β from the lysate abolished SMN import. This effect could be reversed by addition of recombinant importin β. Thus, we conclude that importin β is the cognate import receptor for SMN.

Earlier studies reported that SMN nuclear targeting is regulated by a zinc finger protein, ZPR1 (Gangwani et al., 2001), and demonstrated its presence in a cytoplasmic RNP subcomplex (Narayanan et al., 2002). Notably, the interaction between SMN and ZPR1 is indirect and requires the C termini of both proteins (Gangwani et al., 2001). The data in Figures 3C and 3D reveal that ZPR1 is not essential for SMN import in vitro. Furthermore, the finding that the most prevalent SMA-causing mutation, SMNΔEx7, was fully active for import (Figure 6) suggests that any role played by ZPR1 in SMN import is likely upstream of the actual transport event.

Coupling of SMN and U snRNP Import

We have shown that GFP-SMN transport is disrupted in snRNP-depleted lysate and successfully rescued by purified U snRNPs. These results establish that SMN import in vitro is dependent upon snRNPs. Remarkably, we found that the converse also held true. Import of Cy3-labeled U1 snRNPs was SMN complex dependent, as depleting SMN inhibited U1 import and adding back purified SMN complex restored it. Furthermore, single- and dual-labeling experiments revealed that GFP-SMN import was accelerated by increasing amounts of unlabeled, purified U1 snRNPs. Thus, U snRNPs are rate limiting in SMN import. Finally, both U snRNP and SMN import processes were disrupted by an excess of TMG caps (Figure 5B). Collectively, the data present a strong case for coupled SMN and U snRNP import in somatic cells.

Sm Core Adaptor Complex

U snRNP import involves a bipartite nuclear localization signal composed of the snRNA 5′-TMG cap and the Sm protein core (Fischer et al. 1993; Marshallsay and Lührmann, 1994). Snurportin1 was identified as the adaptor for cap-dependent U snRNP import; however, the Sm core adaptor has eluded identification (Huber et al., 1998, 2002). We found that adding a large molar excess of recombinant snurportin and importin β proteins restored U1 snRNP import defects caused by SMN depletion, presumably through the cap-dependent import pathway. In similar fashion, adding purified, functional SMN complexes restored U1 import in SMN-depleted lysates. Taken together with the presence of the SMN complex in a preimport RNP and its direct interaction with importin β and Sm proteins, we propose that the SMN complex represents the link between the Sm core and importin β (Figure 7). This link to importin β could either be provided by SMN itself or by one of the Gemin proteins (Figure 7). Thus, the SMN complex, or a subset thereof, functions as the adaptor for the Sm-core-dependent U snRNP import pathway.

SMN Import Defects in SMA Patients

U snRNP biogenesis defects correlate with SMA at the molecular level (Paushkin et al. 2002; Meister et al., 2002). SMNΔEx7 is the most common isoform in SMA

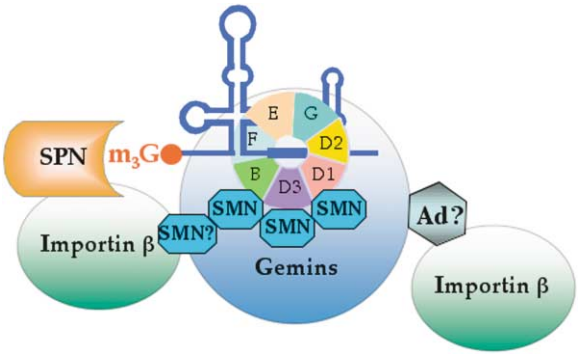

Figure 7. Model of the U snRNP Import Complex

Snurportin1 (SPN) binds the TMG cap (m3G) of U1 snRNA and interacts with importin β. Oligomeric SMN binds methylated, C-terminal tails of Sm-B, -D1, and -D3. Additional members of the SMN complex (Gemins) are required for import but are not specified in the model. The stoichiometry of SMN, importin β, and Gemin proteins has yet to be determined. Whereas SMN can bind directly to importin β in vitro, it is not yet clear whether its binding site is masked while the protein is in the SMN import complex. Thus, in vivo, the connection to importin β may be supplied by SMN itself (SMN?), by an unidentified adaptor protein (Ad?), or by both. The putative adaptor activity could also be supplied by one of the Gemins.

patients. Two recent papers draw apparently conflicting conclusions regarding the functionality of SMNΔEx7. Frugier et al. (2000) described an SMN *nuclear targeting* defect in spinal cords of SMNΔEx7 homozygous mice. In contrast, Zhang et al. (2003) identified a sequence, encoded within exon 7, that seems to comprise a *cytoplasmic retention* element in explanted motor neurons. Frugier et al. (2000) noted, however, that their data fail to distinguish between nuclear import defects versus upstream or downstream events. Thus, the reduced capacity to accumulate SMN in the nucleus could simply be a reflection of preferential SMNΔEx7 isoform degradation. Taken together with our finding that the C-terminal truncation of SMN had little effect on import, these results lead us to propose that SMN is not only actively retained in the cytoplasm but may be released by factors that require exon 7 sequences for activity (e.g., ZPR1).

Strikingly, deletions of the Sm binding Tudor domain (ΔEx3) and the self-oligomerization domain (ΔEx6) abrogated SMN import (Figure 6). Based on our depletion analyses (Figures 2–4), we anticipated the ΔEx3 construct to be import deficient, since the protein fails to bind to Sm protiens. However, the inactivity of the ΔEx6 protein was curious, suggesting a role for oligomerization of SMN in import. A working model of the putative snRNP-SMN import complex is shown in Figure 7. Previously, we showed that members of the SMN complex (e.g., Gemin2 and Gemin3) can be found in cytoplasmic RNP complexes that contain snurportin1 and importin β (Narayanan et al., 2002). Thus, it appears that at least a subset of the Gemins contribute to the Sm-core adaptor complex that interacts with importin β and translocates through the pore (Figure 7).

Perhaps the most tantalizing aspect of our mutational analysis is that certain SMA patient mutations were defective for SMN import (Figure 6). In particular, the defect seen with the E134K missense mutation strongly supports the notion that SMN import defects might underlie SMA pathogenesis in a small subset of patients. While we cannot be certain that the E134K mutant is completely functional for Sm-ring assembly, our biochemical analyses showed that SMN(E134K) binds Sm proteins with high fidelity and completely fails to interact with importin β (Figure 6C). In conclusion, the link between SMN and snRNP import exposes a unique regulatory step in snRNP biogenesis, providing a potential target for SMN dysfunction.

Experimental Procedures

DNA Constructs and In Vitro Translation

GFP-SMN was PCR amplified from the pEGFPC1 vector and TA cloned into pGEM-T (Promega), mutants were created by site-directed mutagenesis, and all constructs were transcribed with an SP6 promoter. Coupled transcription and translation (TnT) kits (Promega) were used as per manufacturer's instructions. The TnT reaction products were analyzed by Western blotting and SDS-PAGE. All the constructs were detected using monoclonal GFP antibodies (Roche).

Antibodies

Antisera used include monoclonal antibody (mAb) ZPR1 (clone LG1), mAb SMN (clone 7B10), mAb U2B″, polyclonal (pAb) Importin β, mAb Sm (clone Y12, Neomarkers), mAb SMN (BD Transduction), mAb and pAb GFP (Roche), and mAb GST and mAb myc (Santa Cruz). Secondary antibodies used were goat α-mouse and goat α-rabbit conjugated horseradish peroxidase (Pierce).

Coimmunoprecipitation

HeLa cells were cultured in DMEM + penicillin and streptomycin, supplemented with 10% FBS (Gibco BRL), and transfected with myc-SMN using Superfect (Qiagen); untransfected HeLa cells served as a control. Cells were harvested 24 hr post-transfection, washed in PBS, and resuspended in 1 ml mRIPA buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1% NP-40, 1 mM EDTA) plus protease inhibitor cocktail tablets (Roche) to lyse cells. Next, the cells were incubated at 4°C for 30 min and centrifuged for 5 min to pellet cellular debris. Where indicated, 5 mg of total protein from the lysate was treated with 1 mg of RNaseA (Sigma) and 5000 units of RNase T1 for 1 hr at 30°C. Twelve microliters of monoclonal α-myc was added to 900 μl of lysate. After incubating for 1 hr, 60 μl of 50% protein G Sepharose beads (Amersham Pharmacia) was added to the lysates and incubated O/N. The beads were then washed 6 times with 1 ml mRIPA, resuspended in 15 μl of 5× SDS loading buffer, boiled, and analyzed by SDS-PAGE. On transfer to nitrocellulose, membranes were probed with α-importin β, followed by incubation with goat α-rabbit conjugated horseradish peroxidase (Pierce) and chemiluminiscent detection (Roche).

Pull-Downs and Immunodepletions

GST pull-downs were conducted by incubating the indicated recombinant proteins with GST-tagged proteins for 1 hr in a buffer containing 450 nM Na^+. Beads were then washed 6× with 1 ml mRIPA, resuspended in 15 μl of 5× SDS loading buffer, boiled, and analyzed by SDS-PAGE. To immunodeplete Sm proteins, SMN, U2B″ from 50 μl of TnT rabbit reticulocyte mix (Promega), and the lysate were incubated at 4°C for 1 hr with 10 μl, 3 μl, and 30 μl of Y12, 7B10, and α-U2B″, respectively. After 1 hr, 60 μl of 50% protein G Sepharose beads (Amersham Pharmacia) was added to the Y12 and 7B10 lysates, and 60 μl of 50% protein-A Sepharose beads (Amersham Pharmacia) was added to the U2B″ reaction. The beads were pelleted by centrifugation. The supernatant and pellets were resuspended in 15 μl of 5× SDS loading buffer, boiled, and analyzed by Western blotting.

To deplete Importin β from the lysate, the GST-tagged fusion of the Importin β binding domain of Importin α (GST-IBB) was used. The lysate was incubated with the GST-IBB beads and centrifuged to pellet the beads. The supernatant and pellet were examined for importin β by Western blotting.

Import Assays

Nuclear import assays were performed with HeLa cells grown to 50%–70% confluency in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin (Gibco-BRL) at 37°C, 5% CO_2 and were subsequently suspended in PBS. After digitonin permeabilization (Adam et al., 1992), GFP-SMN import was studied as described (Dingwall and Palacios, 1998). For Cy3 U1 snRNP import, cells were initially treated as above, and the import assays were processed as described (Huber et al., 1998). To assay GFP-SMN and Cy3-U1 import with the TMG cap analog as competitor, the cells were incubated in P buffer (50 mM HEPES/KOH [pH 7.5], 50 mM KOAc, 8 mM Mg[OAc]2, 2 mM EGTA, 1 mM DTT, and 1 μg/ml each aprotinin, leupeptin, and pepstatin), permeabilized with digitonin for 5 min, and incubated for 5 min at room temperature in P buffer. Cells were then transferred to T buffer (20 mM HEPES/KOH [pH 7.5], 80 mM KOAc, 4 mM Mg[OAc]2, 1 mM DTT, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin) before performing the import reaction.

A standard 25 μl snRNP import reaction contained 0.2 mg/ml tRNA, 0.2 mg/ml BSA, 1 mM ATP, 10 mM creatine phosphate, 50 μg/ml creatine phosphokinase (Roche), 40 nM Cy3-labeled U1 snRNPs, ~100,000 HeLa nuclei, and ~10 mg/ml reticulocye lysate. A standard GFP-SMN import reaction consisted of 1 mM ATP, 5 mM phosphocreatine, 20 U/ml creatine phosphokinase, ~10 mg/ml reticulocyte lysate, ~100,000 HeLa nuclei, 10 mg/ml BSA, 250 mM sucrose, and 0.2 mM GTP. The import assays were conducted at 25°C for 60 min. The Cy3-U1 import assay with purified components was set up with preincubated HeLa cells with 400 ng SMN complexes, 100 ng rImpβ, 1 mM ATP, 5 mM phosphocreatine, 20 U/ml creatine phosphokinase, 0.2 μM Ran, and 1 mM GTP.

Following the import reactions, cells were washed in transport buffer to clear traces of the import reaction, centrifuged onto slides (Cytospin), mounted with antifade, and visualized by a Zeiss Axioplan upright epifluorescent microscope (100× objective). Digital images were taken with a Hamamstsu ORCA-ER C4742-95 CCD camera and Open Lab software (Improvision).

Supplemental Data

Supplemental Data are available at http://www.molecule.org/cgi/content/full/16/2/223/DC1/.

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Note Added in Proof

In Figure S1, mass spectrometric analysis of the indicated band (data not shown) reveals the presence of the Unrip/STRAP protein in the purified SMN complexes obtained by the method of Pellizoni et al. (2002). Consistent with this observation, Unrip/STRAP was previously reported to be a component of the SMN complex by Meister et al. (2001).