SIPP1 (splicing factor that interacts with PQBP1 and PP1) is a widely expressed protein of 70 kDa that has been implicated in pre-mRNA splicing. It interacts with protein Ser/Thr phosphatase-1 (PP1) and with the polyglutamine-tract-binding protein 1 (PQBP1), which contributes to the pathogenesis of X-linked mental retardation and neurodegenerative diseases caused by polyglutamine tract expansions. We show here that SIPP1 is a nucleocytoplasmic shuttling protein. Under basal circumstances SIPP1 was largely nuclear, but it accumulated in the cytoplasm following UV- or X-radiation. Nuclear import was mediated by two nuclear localization signals. In addition, SIPP1 could be piggy-back transported to the nucleus with its ligand PQBP1. In the nucleus SIPP1 and PQBP1 formed inclusion bodies similar to those detected in polyglutamine diseases. SIPP1 did not function as a nuclear targeting subunit of PP1 but re-localized nuclear PP1 to storage sites for splicing factors. The C-terminal residues of SIPP1, which do not conform to a classic nuclear export signal, were required for its nuclear export via the CMR-1 pathway. Finally, SIPP1 activated pre-mRNA splicing in intact cells, and the extent of splicing activation correlated with the nuclear concentration of SIPP1. We conclude that SIPP1 is a positive regulator of pre-mRNA splicing that is regulated by nucleocytoplasmic shuttling. These findings also have potential implications for a better understanding of the pathogenesis of X-linked mental retardation and polyglutamine-linked neurodegenerative disorders.

Nucleocytoplastic Shuttling of the Splicing Factor SIPP1

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SIPP1, also known as SNP70 or Npw38BP, is a polypeptide of 70 kDa that appears to be expressed in all metazoa and higher plants (1–3). It is a largely nuclear protein but has also been reported to be bound to vimentin-containing intermediate filaments in the cytoplasm (2, 3). Within the nucleus SIPP1 is associated with both the speckles (2, 3), which represent storage/assembly sites for splicing factors, and the spliceosomes (3–6), the nuclear protein-RNA complexes that catalyze pre-mRNA splicing. In further agreement with a role in pre-mRNA splicing, SIPP1 was shown to interact directly with single-stranded RNA (2), the polyglutamine-tract-binding protein PQBP1/Npw38 (1), and protein phosphatase-1 (3), all established components of the spliceosomes (Fig. 1A, see also Refs. 6 and 7). RNA binds to the N terminus of SIPP1 (2), the WW-domain of PQBP1 interacts with two proline-rich regions of SIPP1 (1), and the binding of protein phosphatase-1 (PP1) is mediated by two motifs in the central domain of SIPP1 (3). SIPP1 also contains a coiled-coil domain and an Asp-rich domain but the function of these domains is still unknown (1–3).

Although the above data strongly suggest that SIPP1 has a role in pre-mRNA splicing, there are no in vivo data to support this contention. A further complication is that the exact function of the protein ligands of SIPP1 in splicing is not yet known and that these ligands have also functions outside pre-mRNA splicing. Indeed, PP1 plays a role in all major nuclear processes (7), and PQBP1 has also been described as a transcriptional regulator and as an inhibitor of cell growth (8–10). Mutations of PQBP1 have recently been identified as a cause of X-linked mental retardation (11–14), but the underlying molecular mechanism is not understood. Because of its ability to interact with polyglutamine sequences, PQBP1 has also been linked to the pathogenesis of diseases that result from the expansion of polyglutamine sequences, such as spinocerebellar ataxia type 1 (10, 15, 16).

We have started to explore the in vivo role of SIPP1 and show here that SIPP1 functions as a pre-mRNA splicing activator in intact cells. We also demonstrate that SIPP1 is a nucleocytoplasmic shuttling protein and that its nuclear concentration, which appears to be rate-limiting for splicing, is controlled by stress signals and by PQBP1. Our data also provide novel insights into how SIPP1 could be implicated in the pathogenesis of PQBP1-related diseases.

EXPERIMENTAL PROCEDURES

Materials—Polyclonal antibodies against SIPP1 (3) and PQBP1 (10) were raised in rabbits, as described previously. Anti-EGFP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-rabbit TRITC-labeled secondary antibodies were from Sigma. Anti-Histone H3 antibodies were obtained from Abcam. Anti-FLAG antibodies were purchased from Stratagene. Monoclonal anti-hemagglutinin (HA) antibodies were obtained from Sigma-Aldrich.

The indicated SIPP1 mutants and fragments were introduced between the XhoI and BamHI sites of the pEGFP-C1 or pEGFP-N1 plasmids (Clontech), yielding expression vectors for fusions with an N- and C-terminal EGFP tag, respectively. FLAG-SIPP1 was obtained by replacement of the EGFP tag of EGFP-SIPP1 by a FLAG adaptor. All constructs and mutants were verified by DNA sequencing. Expression vectors for 6xHA-PQBP1 (17), Tra2-β1 (18), and the TN24 reporter construct (19) were kind gifts of Dr. E. Golemis, Dr. S. Stamm, and Dr. I. Eperon, respectively.

Cell Culture, Microscopy, and Immunocytochemistry—COS1 cells, HEK293 cells, and HeLa cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. SaOS-2 was grown in McCoy’s
medium supplemented with 10% fetal calf serum. NIH-3T3 was grown in Dulbecco's modified Eagle's medium supplemented with 4.5 g/liter of glucose and 10% fetal calf serum. Transfections were performed with the indicated plasmids and FuGENE 6 (Roche Molecular Biochemicals), according to the instructions of the manufacturer. For immunocytochemistry, 24 h after transfection the cells were washed twice with PBS and fixed for 10 min with 2% formaldehyde. Cell permeabilization was performed by a 10-min incubation in PBS supplemented with 0.5% Triton X-100. The permeabilized cells were washed 3 times for 10 min with PBS, preincubated for 20 min with PBS containing 3% bovine serum albumin, and then incubated for 90 min with polyclonal antibodies against SIPP1, PQBP1, or the FLAG tag. After 3 washes of 10 min with PBS, the cells were incubated for 1 h with secondary anti-rabbit antibodies that were labeled with TRITC (Sigma). Finally, the cells were washed three times for 10 min in PBS. Confocal images were obtained with a Zeiss LSM-510 laser-scanning confocal microscope (Jena, Germany), equipped with the Zeiss Axiovert 100M (plan Apochromat 40× 1.30 numerical aperture oil immersion objective) and using the standard fluorescein isothiocyanate-TRITC filter set.

For the heterokaryon experiments, HeLa cells were used as donor cells and NIH-3T3 cells as acceptor cells. 24 h after transfection of the HeLa cells with EGFP-SIPP1 or NL5.4β-galactosidase-EGFP, the cells were seeded with the same amount of NIH-3T3 cells on a sterile Lab-Tek™ Chambered Coverglass (Nunc) and allowed to adhere for 3 h at 37°C. Subsequently, 100 μM cycloheximide (Sigma) was added to block translation. After 3 h cell fusion was induced by the addition of 45% polyethylene glycol 3350 (Sigma) for 2 min at 37°C. Subsequently, the cells were washed 3 times with PBS and further incubated in culture medium for more hours. Finally, the cells were fixed with 2% formaldehyde for 5 min and stained with Hoechst for 10 min. After washing for 5 min, the EGFP fusions were visualized by fluorescence microscopy (Olympus Biosystems CELL®).

**FIGURE 1.** Domain structure and subcellular localization of SIPP1. A, the large horizontal bar represents SIPP1. The SIPP1 domains and interaction sites for PP1 and PQBP1 are indicated. B, subcellular localization of SIPP1 and EGFP fusions. Hela cells were stained for endogenous SIPP1 with anti-SIPP1 antibodies. HEK293 cells were transfected with either EGFP-SIPP1 or SIPP1-EGFP, and the fluorescence was visualized after 24 h by confocal microscopy.

**FIGURE 2.** Mapping of the determinants for the nuclear targeting of SIPP1. A, sequence of SIPP1 showing the predicted nuclear localization signals (NLS, in red) and nuclear export signals (NES, in blue). B, the indicated mutants of EGFP-SIPP1 were transiently expressed in HEK293 cells, and their subcellular localization was analyzed by confocal laser microscopy 24 h after transfection. The mutations (m) of the NLSs are as follows: mNLS1, K31A/K32A/K34A/K35A; mNLS2, K81A/R82A; mNLS3, R228A/R230A; and mNLS4, K319A/K320A. Identical results were obtained in COS1 cells (not shown).

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**A. Consensus NLSs and NESs in SIPP1**

| Consensus NLS | NES |
|---------------|-----|
| MORRSTSSSTSSGPMNPSPQDKEAKRRTELNNKFGVRAVYLMKEDPQIIVDRGKLDKEMPNFQQ | 70 |
| PQGMLNEKVLGDEFLRETVFIRLRLVEKNNITQQQKLEYVQRTQRAQLQCVFDVAVNQKQVYES | 140 |
| LFDPAHPSNMLGIQDGLPGQAPPSILKETSATGPFARASVSLILQGINVRFLOPSGKEPPPGQPPQ | 210 |
| VLGQYGRKVLPPDLPPPPDEMLYSPFLAQRGHECDMTSTSDQYFEDMWXDAEHTERSDOSDTRDS | 280 |
| AESQDDFQHREDEONQNDDKQGLRVFMAPFLQK | 350 |
| RKSEPHEDDDSDEEAEKQEQKQKHDADSDSTAASQGQQPPQPSAPAQCQAPMPQPPGLPAPP | 420 |
| LRPPQGTPPLLGPQPPGPQAPPFLRPQGFQERFLEPLLPPQPPQPPQFRQPPGPQPPPPFR | 490 |
| LPPAPPQGPPQPPQPPQPPQPPQPPQPPQPPQPPQPPQPPQPPQGLPPPPFLQK | 560 |
| TISASQPQITNPFAEVTPATLAVRVEKNGATAVQPQREDDSEAPVKAAXAPRSGPSVAVSVQ | 630 |
| BAFHKEQHULL | 641 |

**B. Mutation of putative NLSs in SIPP1**
Nucleocytoplasmic Shuttling of SIPP1

In Vitro Nuclear Export Assay—The adopted protocol was based on that of Pearson et al. (20). HeLa cells were permeabilized with 40 μg/ml digitonin (Sigma) for 5 min in transport buffer containing 20 mM Hepes at pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EDTA, 2 mM dithiothreitol, 0.5 mM benzamidine, and 5 mM leupeptin. Cells were washed three times with ice-cold transport buffer to wash out all the cytoplasmic factors. Subsequently, an ATP regeneration system (1 mM ATP, 0.2 mM GTP, 5 mM creatine phosphate) was added as well as 10% (v/v) of a cytoplasmic S100 fraction (Cil-Biotech), and aliquots of the soluble and nuclear fractions were taken at the indicated times during incubation at 30 °C in a humidified chamber. The aliquots were analyzed for SIPP1 and Histone H3 by immunoblotting.

In Vivo Splicing Assays—These were performed essentially as described by Nasim et al. (19). Briefly, HEK293 cells were transfected with the reporter gene TN24 and the indicated plasmids. 48 h after transfection the cells were lysed using the passive lysis buffer (Promega Corp., Madison, WI). An aliquot of the lysate was used to measure luciferase activity using the assay system from Promega. The β-galactosidase activity was measured with o-nitrophenyl-β-D-galactopyranoside as a substrate. The expression of the constructs in the lysates was verified by Western blot analysis with anti-EGFP antibodies.

RESULTS

Determinants of the Nuclear Translocation of SIPP1—Under basal conditions endogenous SIPP1 was nearly completely nuclear in all tested cell types, including HeLa (Fig. 1B), SaOS-2, U2OS, COS1, and 293 cells (not shown). SIPP1 with an N-terminal EGFP tag (EGFP-SIPP1) was also exclusively nuclear, but, surprisingly, SIPP1 with a C-terminal EGFP tag (SIPP1-EGFP) was largely cytoplasmic (Fig. 1B). Thus, endogenous SIPP1 and EGFP-SIPP1 are both nuclear. The size of the latter fusion protein (∼95 kDa) precludes a passive nuclear transport. Consistent with an active nuclear transport, SIPP1 has been found to harbor four consensus polybasic nuclear localization signals (1), termed NLS1–4 in Fig. 2A, one of which (NLS1) has previously already been identified as a functional NLS (3). Mutation of NLS1 (K31A/K32A/K34A/K35A) or NLS2 (K81A/R82A) clearly resulted in a cytoplasmic accumulation of EGFP-SIPP1, but mutation of NLS3 (R228A/R230A) or NLS4 (K319A/K320A) did not have such an effect (Fig. 2B). Unexpectedly, the combined mutation of all four predicted NLSs only partially abolished the nuclear accumulation of SIPP1 (Fig. 2B), indicating that SIPP1 also contains non-canonical NLSs or can also be transported to the nucleus by association with an NLS-containing polypeptide.

To test whether SIPP1 and its ligand PQBP1 can be co-transported to the nucleus, we made use of the observation that SIPP1-EGFP is cytoplasmic (Fig. 1B) and that EGFP-SIPP1-mNLS1/2/3/4 is partially cytoplasmic (Fig. 2B). Both EGFP-SIPP1-mNLS1/2/3/4 (Fig. 3A) and SIPP1-EGFP (Fig. 3B) could be re-targeted to the nucleus by the co-expression of PQBP1. This re-targeting was not seen following the co-expression of PQBP1-W75A/P78G, which is mutated in its WW-domain and does not interact with SIPP1 (Fig. 3C). These data are consistent with the view that SIPP1 and PQBP1 are co-transported and/or that SIPP1 is a nucleocytoplasmic shuttling protein and is retained in the nucleus by binding to PQBP1. Interestingly, SIPP1 and PQBP1 co-localized in nuclear inclusion bodies and within these inclusion bodies SIPP1-EGFP was enriched in the core, whereas PQBP1 accumulated preferentially at the edges (Fig. 3D). A similar distribution within nuclear inclusion bodies has previously been noted after the co-expression of PQBP1 and its ligands ataxin-1 or USP–15 kDa (10, 17).

SIPP1 cannot be piggy-back transported to the nucleus by association with PP1, because the latter does not contain a functional NLS (21). PP1 itself is transported to the nucleus by association with proteins that contain an NLS as well as a PP1-binding motif of the RVXF-type, which conforms to the consensus sequence [RK]-X<sub>0</sub>...[VI]-[P]-[FW], where
SIPP1 was not able to target EGFP-PP1 to the nucleus by fusion with the NLS of the SV40 antigen. Thirdly, SIPP1 was exported from the nucleus using an in vitro nuclear export assay (Fig. 6A). In this assay, digitonin-permeabilized HeLa donor cells were supplemented with the cytoplasmic S100 fraction and an ATP-regenerating system. At the indicated times, the release of SIPP1 to the cytoplasmic fraction was detected by immunoblotting. Under these circumstances SIPP1 was released from the nuclei in a time-dependent manner (Fig. 6C, right panel). However, SIPP1 was not released in the absence of ATP and the S100 fraction (Fig. 6C, left panel). Moreover, Histone H3, which does not shuttle between the nucleus and the cytoplasm, was not.

**SIPP1 Shuttles between the Nucleus and the Cytoplasm**—SIPP1 is largely nuclear but can occasionally also be detected in the cytoplasm (2, 3). This prompted us to examine whether the subcellular distribution of SIPP1 is subject to regulation. We found that an exposure of SaOS-2 cells (Fig. 5) or HeLa cells (not shown) to X-radiation (5 Gy and 2.9 Gy/min) or UV-radiation (30 mJ/cm²) consistently resulted in a cytoplasmic accumulation of endogenous SIPP1 after 16 and 24 h, respectively, pointing to the existence of a nuclear export mechanism for SIPP1. In contrast, the localization of NIPP1, another nuclear interactor of PP1, was not affected by UV- or X-irradiation (not shown). Various lines of experimental evidence subsequently indicated that SIPP1 is a nucleocytoplasmic shuttling protein. Firstly, SIPP1-EGFP, which is nearly exclusively cytoplasmic, accumulated in the nucleus after the addition of 20 nM leptomycin B, an inhibitor of nuclear export via the CRMP1 receptor (Fig. 6A). EGFP-SIPP1-mNLS1/2/3/4, which is partially cytoplasmic (Fig. 2B), was also nearly completely nuclear after the addition of leptomycin B (Fig. 6A). Secondly, in interspecies heterokaryon assays we found that EGFP-SIPP1, expressed in HeLa donor cells, migrated to the nuclei of NIH-3T3 receptor cells within 3 h after the cell fusion with polyethylene glycol and, in the presence of cycloheximide, to block new protein synthesis (Fig. 6B). The murine NIH-3T3 cells can be easily differentiated from the human HeLa cells by the presence of heterochromatin clumps in the nucleoplasm stained by Hoechst 33342. No such migration was seen for β-galactosidase that was targeted to the nucleus by fusion with the NLS of the SV40 antigen. Thirdly, SIPP1 was exported from the nucleus using an in vitro nuclear export assay (Fig. 6D). This prompted us to examine whether the subcellular distribution of SIPP1 is subject to regulation. We found that an exposure of SaOS-2 cells (Fig. 5) or HeLa cells (not shown) to X-radiation (5 Gy and 2.9 Gy/min) or UV-radiation (30 mJ/cm²) consistently resulted in a cytoplasmic accumulation of endogenous SIPP1 after 16 and 24 h, respectively, pointing to the existence of a nuclear export mechanism for SIPP1. In contrast, the localization of NIPP1, another nuclear interactor of PP1, was not affected by UV- or X-irradiation (not shown). Various lines of experimental evidence subsequently indicated that SIPP1 is a nucleocytoplasmic shuttling protein. Firstly, SIPP1-EGFP, which is nearly exclusively cytoplasmic, accumulated in the nucleus after the addition of 20 nM leptomycin B, an inhibitor of nuclear export via the CRMP1 receptor (Fig. 6A). EGFP-SIPP1-mNLS1/2/3/4, which is partially cytoplasmic (Fig. 2B), was also nearly completely nuclear after the addition of leptomycin B (Fig. 6A). Secondly, in interspecies heterokaryon assays we found that EGFP-SIPP1, expressed in HeLa donor cells, migrated to the nuclei of NIH-3T3 receptor cells within 3 h after the cell fusion with polyethylene glycol and, in the presence of cycloheximide, to block new protein synthesis (Fig. 6B). The murine NIH-3T3 cells can be easily differentiated from the human HeLa cells by the presence of heterochromatin clumps in the nucleoplasm stained by Hoechst 33342. No such migration was seen for β-galactosidase that was targeted to the nucleus by fusion with the NLS of the SV40 antigen. Thirdly, SIPP1 was exported from the nucleus using an in vitro nuclear export assay (Fig. 6D).
Nucleocytoplasmic Shuttling of SIPP1

A. Effect of leptomycin B

FIGURE 6. SIPP1 shuttles between the nucleus and the cytoplasm. A, 24 h after transfection of COS1 cells with either SIPP1-EGFP or EGFP-SIPP1-mNLS1/2/3/4, vehicle or 20 nM leptomycin B (LB) were added. 3 h later the subcellular distribution of the EGFP fusions was analyzed by confocal microscopy. B, heterokaryon experiments. HeLa cells (donor cells) transfected with EGFP-SIPP1 or NLSSV40-galactosidase-EGFP (NLSSV40-gal) were seeded with non-transfected NIH-3T3 cells (acceptor cells). The cells were incubated for 3 h with 100 µM cycloheximide, an inhibitor of translation. The EGFP-tagged fusions were visualized by fluorescence microscopy, and the nuclei were stained with Hoechst 33258. The murine NIH-3T3 cells can be easily differentiated from the human HeLa cells by the presence of heterochromatin clumps in the nucleoplasm (arrows). C, in vitro export assay. Digitonin-permeabilized HeLa cells were supplemented with either an S-100 cytoplasmic fraction plus an ATP regeneration system or transport buffer alone. At the indicated times, aliquots of the soluble and nuclear fraction were taken for immunoblotting with anti-SIPP1 and anti-Histone H3 antibodies.

B. Heterokaryon assays

C. In vitro export assay

Determinants of the Nuclear Export of SIPP1—The inhibition of the nuclear export of SIPP1 by leptomycin B (Fig. 6A) strongly suggests an involvement of the CRM1-export pathway and also indicates that SIPP1 may contain a nuclear export signal (NES). SIPP1 contains three sequences, termed NES1–3 in Fig. 2A, that could qualify as hydrophobic NES motifs (22). However, these predicted NESs do not appear to function as such because the mutation of NES1 (L110A/L112A), NES2 (I156A/L158A), or NES3 (L330A/M333A/L335A) did not result in a nuclear accumulation of SIPP1-EGFP (Fig. 7A). Further investigations revealed, however, that the deletion of C-terminal sequences of SIPP1, as in SIPP1-(Δ559–641)-EGFP (not shown), SIPP1-(Δ601–641)-EGFP (not shown), and SIPP1-(Δ633–641)-EGFP (Fig. 7B), re-targeted this fusion to the nucleus, suggesting that the C-terminal 9 residues of SIPP1 are essential for its nuclear export. The sequence of the C-terminal 9 residues of SIPP1 is conserved in metazoas (Fig. 7D) and shows some similarities with classic, hydrophobic NESs, including the C-terminal two leucines. Mutation of the latter leucines into an alanine was indeed sufficient to re-target SIPP1-EGFP to the nucleus (Fig. 7B).

In further agreement with the notion that the C terminus of SIPP1 contains a NES we found that EGFP, which accumulates to a similar concentration in the cytoplasm and the nucleus, was largely cytoplasmic when EGFP was C-terminally fused to the last 82 residues of SIPP1 (Fig. 7C). However, fusion of the last 9 residues of SIPP1 to EGFP did not affect its subcellular distribution, indicating that sequences N-terminal to these 9 residues are also important for nuclear export and/or that the fusion with EGFP hampers the interaction of the NES with the CRM1 receptor.

The Nuclear Concentration of SIPP1 Is Rate-limiting for pre-mRNA Splicing—Consistent with a role for SIPP1 in pre-mRNA splicing (see the introduction), we found that immunoprecipitates of SIPP1 from HeLa cell nuclear extracts contain all the small nuclear RNAs involved in splicing, i.e. U1, U2, U4, U5, and U6 as well as a large number of established splicing factors (not shown). To examine whether SIPP1 also plays a role in pre-mRNA splicing in vivo, we have used the dual-reporter system described by Nasim et al. (19). The reporter construct contains sequences encoding β-galactosidase as well as luciferase, and these sequences are separated by an intronic sequence derived from the adenovirus and human αs-tropomyosin genes (Fig. 8A). Importantly, the intron contains three translation stop signals. As a consequence, when the transcript is not spliced, it gives rise to β-galactosidase, whereas a fusion of β-galactosidase and luciferase is generated after splicing. Thus, the luciferase/β-galactosidase ratio is a measure of splicing efficiency. The expression of Tra2-β1, an established splicing activator (18), increased the luciferase/β-galactosidase ratio by some 100%, as did the expression of EGFP-SIPP1 (Fig. 8B). Interestingly, SIPP1-EGFP, which is largely cytoplasmic (Fig. 1B), was also a less potent splicing activator than the nuclear EGFP-SIPP1. However, SIPP1-(Δ633–641)-EGFP, which is completely nuclear (Fig. 7B), was an equally potent activator of splicing as was EGFP-SIPP1. SIPP1 fragments, such as EGFP-SIPP1-(1–372), EGFP-SIPP1-(180–372), and EGFP-SIPP1-(359–641), did not affect the splicing of the reporter construct. Finally, EGFP-SIPP1-(45–641), which lacks NLS1 and is partially cytoplasmic (3), was also a relatively poor activator of splicing. It was released from the nuclei, whether or not the S100 fraction and ATP were present.
verified that all SIPP1 fusions and fragments were expressed in the HEK293 cells (Fig. 8C). The above data indicated that the nuclear concentration of SIPP1 is limiting for pre-mRNA splicing in HEK293 cells. In further agreement with this notion, we found that the splicing activation by SIPP1-EGFP was 33% higher following its re-targeting to the nucleus by the co-expression of PQBP1 (Fig. 8B). In contrast, the co-expression of PQBP1-W75A/P78G, which did not affect the subcellular distribution of SIPP1 (Fig. 3B), diminished the splicing activation by SIPP1-EGFP by 27%. The decreased splicing stimulation may be explained by competition of PQBP1-W75A/P78G with endogenous PQBP1.

**DISCUSSION**

**SIPP1 Is a Splicing Activator**—We show here for the first time that SIPP1 functions as a splicing activator in vivo (Fig. 8). Indeed, SIPP1 enhanced the splicing efficiency of a reporter gene up to 2-fold, an effect that was not seen with various SIPP1 fragments. Also, SIPP1 mutants with a predominant cytoplasmic accumulation were much less effective splicing activators, indicating that it is the nuclear pool of SIPP1 that affects splicing. Furthermore, the targeting of SIPP1-EGFP to the nucleus by the co-expression of PQBP1 (Fig. 3B) was also correlated with an increased splicing efficiency. How does SIPP1 promote pre-mRNA splicing? Makarova et al. (6) found that SIPP1 and PQBP1 were stably associated with a pre-spliceosomal complex, termed BΔU1, that had not yet undergone catalytic activation. Intriguingly, these proteins were not detected in affinity-purified activated spliceosomes, but this could simply be a reflection of their less stable association with the mature spliceosomes, because the latter were affinity-purified under rather stringent conditions (presence of heparin). In accordance with this view, immunoprecipitation experiments suggested that SIPP1 remains associated with the spliceosomes throughout splicing (3). In addition to a function in spliceosome assembly and/or activation, SIPP1 could also play a role in splicing catalysis per se, as suggested by the observed inhibition of the second catalytic step of splicing after the addition of SIPP1-(180–372) to HeLa cell nuclear extracts (3). This splicing inhibition could not be explained by a displacement of SIPP1.
from the spliceosomes, suggesting that this SIPP1 fragment somehow inhibits or titrates a splicing factor that is required for the second step of splicing. Finally, SIPP1 could affect splicing by its ability to target specific splicing factors for dephosphorylation by PP1 (Fig. 4). Spliceosome disassembly and the re-targeting of some splicing factors to the speckles after splicing has indeed been associated with dephosphorylation events (7). Some splicing factors have also functions in other processes, such as transcription (23). However, it currently seems unlikely that SIPP1 has functions outside splicing, because all known SIPP1 ligands are splicing factors.

**Nucleocytoplasmic Shuttling of SIPP1**—A surprising finding was that the mutation in SIPP1 of all four consensus NLSs only partially abolished its nuclear targeting (Fig. 2B). This indicates that SIPP1 can also be translocated to the nucleus by co-transport with another nuclear protein, possibly PQBP1 (Fig. 3). It is tempting to speculate that the relative contribution of these mechanisms for nuclear transport of SIPP1 is subject to regulation and varies, for example, with the cellular concentration of PQBP1. Thus, the nuclear translocation of SIPP1 in certain brain regions, such as the cerebellar cortex and hippocampus, where the level of PQBP1 is relatively high (9), could be largely PQBP1-dependent. It is also intriguing that various mutations of PQBP1 that have been associated with X-linked mental retardation, result in the expression of PQBP1 forms that lack its putative NLS (10–12). Because PQBP1 and SIPP1 can be co-transported to the nucleus, this indicates that (some of) the effects of these mutations of PQBP1 could be accounted for by a deficient nuclear accumulation of SIPP1. It will therefore be interesting to examine the subcellular distribution of SIPP1 in cells from patients with PQBP1-linked mental retardation.

PQBP1 interacts with polyglutamine tracts that are present in various proteins, such as ataxin-1, and this interaction is increased by expanded polyglutamine sequences that are associated with neurodegenerative disorders like spinocerebellar ataxia type-1 (10). One consequence of the increased PQBP1-ataxin-1 interaction is an enhanced binding of PQBP1 to the C-terminal domain of the largest subunit of RNA polymerase-II, and this leads to a reduction in the level of phosphorylated RNA polymerase-II and hence a decreased transcription. Because SIPP1 and RNA polymerase-II both interact with the WW-domain of PQBP1, it can be envisaged that the binding of PQBP1 to extended polyglutamine

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**FIGURE 8. SIPP1 is an activator of pre-mRNA splicing in vivo.** A, structure of the reporter construct. The β-galactosidase and luciferase genes are fused in-frame, but are separated by an intronic sequence with three in-frame stop codons. In the absence of splicing β-galactosidase is generated, whereas an active fusion of β-galactosidase and luciferase is generated after splicing of the primary transcript. B, HEK293 cells were transiently transfected with the reporter plasmid (TN24) and the indicated expression plasmids. The graph shows the % stimulation of splicing, as derived from the luciferase/β-galactosidase ratio, as compared with the ratio obtained with EGF. The data represent the means ± S.E. (n = 3) and are representative for five different experiments. C, immunoblot with anti-EGFP antibodies showing expression of the EGFP fusions in HEK293 cells and with anti-PQBP1 antibodies showing the expression of endogenous PQBP1, 6HA- PQBP1, and 6HA-PQBP1-W75A/P78G.
sequences also enhances the PQBP1-SIPP1 interaction and thereby interferes with the function of SIPP1 in splicing. Polyglutamine-tract-linked neurodegenerative diseases are also characterized by nuclear aggregates that are formed by the extended polyglutamine tracts. Intriguingly, the co-expression of PQBP1 and one of its ligands, be it U5–15 kDa (17), ataxin-1 (10), or SIPP1 (Fig. 3D), results in the formation of nuclear inclusion bodies that are similar to those nucleated by the protein products of polyglutamine diseases genes. In all cases, PQBP1 envelopes these inclusion bodies while the PQBP1 ligands form the core, indicating that the PQBP1 ligands seed the inclusions. Collectively, these data suggest that an overexpression of PQBP1 ligands, including SIPP1, potentially contributes to the pathology of polyglutamine-linked neurodegenerative diseases.

The nuclear export of SIPP1 is mediated by its C terminus, as indicated by the nuclear accumulation of SIPP1-EGFP after deletion or mutation of the C-terminal residues (Fig. 7B). SIPP1-EGFP also accumulates in the nucleus after the addition of leptomycin B (Fig. 6A), an inhibitor of the CRM1 exporter, indicating that the C terminus of SIPP1 harbors a NES. CRM1 directs nuclear export of target proteins via direct binding to leucine/hydrophobic stretches of amino acids. However, the exact spacing of the leucine/hydrophobic patch is variable, making it difficult to identify CRM1 binding sites (24). Although the C terminus of SIPP1 contains conserved hydrophobic residues and does qualify as a potential NES, we cannot be certain that it actually functions as an NES, because the C-terminal 9 residues did not drive the nuclear export of a fused heterologous protein (Fig. 7C). It is possible, however, that the putative NES fused to the heterologous protein was not accessible for interaction with CRM1. In accordance with this view, we noted that larger C-terminal fragments of SIPP1 did drive the nuclear export of EGFP.

Under basal circumstances SIPP1 is largely nuclear, indicating that import is much faster than export. However, this ratio changes during both X- and UV-irradiation, resulting in a cytoplasmic accumulation of SIPP1. The mechanism for this altered equilibrium is not clear and can be the result of either a decreased import rate and/or an increased export rate. The nucleocytoplasmic shuttling of some proteins is controlled by reversible phosphorylation (24, 25). Similar to what has been recently described for cyclin D1 (24), we have contemplated the idea that the cytoplasmic accumulation of SIPP1 after irradiation is caused by phosphorylation, resulting in an exposure of its C-terminal NES. The C terminus of SIPP1 contains a conserved tyrosine (Tyr-630). However, mutation of this tyrosine into a phenylalanine or an aspartic acid did not affect the subcellular localization of SIPP1-EGFP (not shown), which is not suggestive for a role of phosphorylation of Tyr-630. Also, mutation of the PP1 binding sites did not affect the subcellular localization of EGFP-SIPP1 (not shown), SIPP1-EGFP (not shown), or FLAG-SIPP1 (Fig. 4C), indicating that PP1 is not implicated in the nucleocytoplasmic shuttling of SIPP1.

In conclusion, we have shown here that SIPP1 is a pre-mRNA splicing activator in vivo that is likely to be present at limiting concentrations. The function of SIPP1 appears to be regulated by nucleocytoplasmic shuttling, but further investigations are needed to understand the underlying regulatory mechanisms.

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