Hypophosphorylated ASF/SF2 Binds TAP and Is Present in Messenger Ribonucleoproteins*

Ming-Chih Lai and Woan-Yuh Tarn‡

From the Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan

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Serine/arginine-rich proteins (SR proteins) function in precursor mRNA (pre-mRNA) splicing and may also act as adaptors for mRNA export. SR proteins are dynamically phosphorylated in their RS domain, and differential phosphorylation modulates their splicing activity and subcellular localization. In this study, we investigated the influence of phosphorylation on the function of SR proteins in events occurring during mRNA maturation. Immunoprecipitation experiments showed that the mRNA export receptor TAP associates preferentially with the hypophosphorylated form of shuttling SR proteins, including ASF/SF2. Overexpression of ASF induced subnuclear relocation of TAP to SR protein-enriched nuclear speckles, suggesting their interaction in vivo. Moreover, the ASF found in a nucleoplasmic fraction rich in heterogeneous nuclear ribonucleoprotein (hnRNP) complexes is hyperphosphorylated, whereas mature messenger RNP (mRNP)-bound ASF is hypophosphorylated. Therefore, hypophosphorylation of ASF in mRNPs coincides with its higher affinity for TAP, suggesting that dephosphorylation of ASF promotes both its incorporation into mRNPs and recruitment of TAP for mRNA export. Thus, the phosphorylation state of RS domains may modulate the function of mammalian shuttling SR proteins during mRNA maturation or export.

Eukaryotic mRNAs are synthesized and processed in the nucleus and subsequently exported to the cytoplasm for protein translation. Nuclear export of mRNA is generally mediated by a pathway that is largely distinct from those used to export non-coding RNAs such as tRNAs, rRNAs, and small nuclear RNAs (1). During transcription, mRNAs are packaged into complexes with various heterogeneous nuclear ribonucleoproteins (hnRNPs). Some of the hnRNPs may participate in precursor mRNA (pre-mRNA) splicing or mRNA export/localization (2, 3). Upon completion of mRNA processing, export factors join and accompany messenger RNP complexes (mRNPs) for subsequent exit from the nucleus (1–3). The most thoroughly characterized mRNA export receptor is TAP, which interacts directly with the nuclear pore complex (4). Evidence implicating TAP in mRNA export initially came from a study of nuclear export of unspliced simian type D retroviral mRNAs that contain the constitutive transport element (CTE) (5). TAP promotes the nuclear export of CTE-bearing RNAs via direct binding to the CTE (5). Microinjection of excess CTE-containing RNAs into Xenopus oocytes blocks nuclear export of cellular mRNAs; this blockade can be rescued by injection of recombinant TAP (5, 6). Depletion of endogenous TAP from cultured Drosophila cells results in a robust accumulation of poly(A)+ RNAs within the nucleus, suggesting an essential role for TAP in mRNA export (7). Yeast Mex67p, an ortholog of mammalian TAP, is also involved in bulk poly(A)+ RNA export (8), indicating that TAP has a conserved role as a general mRNA export factor. TAP interacts with p15, a protein related to nuclear transport factor 2 (NTF-2); formation of the TAP/p15 heterodimer is required for efficient nuclear pore complex binding and significantly enhances the ability of TAP to export mRNAs from the nucleus (4). TAP-mediated cellular mRNA export requires various adaptor proteins that associate with TAP and mRNP cargoes (2–4).

In metazoan cells, the majority of primary transcripts undergo splicing to become mature mRNAs. Experiments with Xenopus oocytes earlier demonstrated a role for splicing in facilitating mRNA export (9). Intron removal results in deposition of a mRNP complex, termed the exon junction complex (EJC), at a region immediately upstream of the splice junctions of the mature mRNA (10). Several of the EJC components shuttle between the nucleus and cytoplasm and interact directly with TAP (2). The EJC factor REF is posibly recruited to spliced mRNAs by UAP56, a putative RNA helicase involved in early spliceosome formation (4). These observations support a direct coupling of pre-mRNA splicing and mRNA export. However, splicing dependence of mRNA export is argued by recent demonstrations that depletion of individual EJC components had no profound effect on bulk mRNA export (11) and that splicing only modestly enhanced nuclear mRNA export in metazoan cells (12, 13). Nonetheless, the EJC may still function in assembly or maturation of export-competent mRNPs (2, 3).

SR proteins are a family of serine/arginine dipeptide-rich proteins involved in both constitutive and regulated pre-mRNA splicing (14). Some of the SR protein family members shuttle between the nucleus and cytoplasm, suggesting their role in mRNA export (15). A recent finding shows that the shuttling SR proteins 9G8 and SRp20 interact directly with TAP to promote the nuclear export of mRNA (16, 17). The yeast RNA-binding protein Npl3p, a close relative of metazoan SR proteins, is also involved in mRNA export (18). Npl3p is phosphorylated by the cytoplasmic SR protein kinase Sky1p (19). Npl3p accumulates in the cytoplasm and remains bound to poly(A)+ RNA in sky1 mutants, suggesting that phosphorylation of Npl3p promotes either its dissociation from exported mRNPs or its nuclear re-import (19). Analogously, cellular localization of mammalian

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SR proteins is regulated through phosphorylation of their RS domains (14). RS domain phosphorylation differentially influences the interaction of SR proteins with their import receptors and thereby modulates their nuclear import (20, 21). Nevertheless, whether phosphorylation also regulates nuclear export of SR proteins remains an unanswered question.

In this report, we provide evidence that hypophosphorylated ASF, a shuttling SR protein, binds TAP and associates with mature mRNPs. Thus, dephosphorylation of SR proteins is possibly critical for their nuclear export or post-splicing functions.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction.**—The cDNA of human TAP (NXF1) (kindly provided by J. A. Steitz, Yale University) was inserted into pcDNA3.1 (Invitrogen), yielding an expression vector for the C-terminally FLAG-tagged TAP. The cDNAs of 9G8, ASF, and SC35 were obtained by reverse transcription (RT)-PCR using HeLa cell RNAs as template, and each was then inserted into pCEP4 (Invitrogen) to generate the constructs encoding C-terminally HA-tagged SR proteins. Plasmid pEGFP-ASF was constructed by subcloning the ASF cDNA fragment into pEGFP-C1 (Clontech).

**Cell Culture and Transfection.**—Human HeLa or 293 cells were cultured and transfected using LipofectAMINE 2000 (Invitrogen) as described previously (22). To establish FLAG-TAP-expressing cell lines, 293 cells were transfected with the FLAG-TAP expression vector and cultured in the presence of 300 μg/ml G418 (Clontech) for 2 weeks. Resistant colonies were picked and selection with G418 continued for an additional 2 weeks. The surviving cells were screened for stable expression of FLAG-TAP using Western blotting.

**Immunoprecipitation.**—FLAG-TAP was co-expressed with one of the HA-tagged SR proteins (9G8, ASF, or SC35) in 293 cells for 2 days. The cell lysates were prepared and treated with RNase A according to Lykke-Andersen et al. (23). The lysates of ~1 × 10^6 cells in 0.5 ml were incubated with 20 μl of anti-FLAG-coupled agarose (M2; Sigma) for immunoprecipitation. The precipitates were analyzed by Western blotting with monoclonal anti-HA antibody (a gift from S.-C. Cheng, Academia Sinica, Taipei, Taiwan). In vitro phosphorylation of the precipitated proteins was performed using 50 ng of recombinant GST-SRPK1 in a 20-μl reaction as described (22). Dephosphorylation of cell lysate proteins was performed in a 20-μl reaction containing 5 μl of the lysate, 40 units of alkaline phosphatase (Roche Applied Science) or 800 U of λ protein phosphatase (BioLabs) at 30 °C for 45 min.

**In Vitro Pull-down Assay.**—Recombinant GST-ASF was prepared and phosphorylated by GST-SRPK1 in vitro as described (22); ATP was omitted in the mock-phosphorylation reaction. For the pull-down experiments, FLAG-TAP was obtained from stably expressing 293 cells (see above) and used as bait. The FLAG-TAP-containing lysate (1 ml) was prepared (23) from ~1 × 10^6 cells and then incubated with 20 μl of anti-FLAG-coupled agarose (M2; Sigma). After extensive washing with NET-2 buffer (23), TAP-bound beads were incubated with 1 μg of phosphorylated or mock-treated GST-ASF at 4 °C for 1 h. Subsequently, the beads were washed with NET-2 buffer, and bound proteins were analyzed by immunoblotting with anti-GST antibody (Upstate Biotechnology).

**Subcellular Fractionation.**—Subcellular fractionation was performed essentially as described by Mili et al. (24). Approximately 2 × 10^7 of mock- or pCPE4-HA-ASF-transfected 293 cells were collected in 1 ml of RSB-100 buffer (24). After centrifugation, the cell pellet was resuspended in RSB-100 containing 0.5% Triton X-100 and phosphatase inhibitors (10 μM KF and 5 μM β-glycerophosphate). Following incubation on ice for 10 min, the suspension was centrifuged at 1,000 × g for 5 min. The Triton X-100-solubilized supernatant was collected and the pellet was resuspended in the same buffer and sonicated twice by 5-s bursts. The sonicated material was incubated on ice for 10 min followed by centrifugation, and the supernatant was collected (the Triton X-100-insoluble fraction). To immunoprecipitate RNPs, we used 25 μl of anti-HA antibody in the presence of RNase A. Detection of transiently expressed SR proteins revealed the direct interaction of FLAG-TAP with the two shuttling SR proteins (Fig. 1A, lanes 1–4), consistent with a previous report (17). In contrast, SC35 did not co-precipitate with FLAG-TAP (lanes 5 and 6). However, immunoprecipitated 9G8 and ASF showed higher electrophoretic mobility on SDS-PAGE than the corresponding lysate proteins (lanes 1–4, compare IP to input). Since ASF co-precipitated most efficiently (Fig. 1A), and its association with TAP was also observed by mass spectrometric analysis of TAP-interacting proteins (data not shown), we next examined whether the faster migrating ASF corresponds to a dephosphorylated form.

To completely remove the phosphate groups of ASF, an HA-tagged ASF-containing cell lysate was treated with λ protein phosphatase or alkaline phosphatase. Phosphatase-treated more RNA than the insoluble fraction, equal amounts of total RNAs from each fraction were subjected to RT-PCR analysis.

**Indirect Immunofluorescence.**—The pcDNA-FLAG-TAP vector was transfected alone or with pEGFP-ASF into HeLa cells for 40 h. Indirect immunofluorescence staining was performed as described previously (22). After fixation, the cells were stained with monoclonal anti-FLAG (Sigma) followed by rhodamine-conjugated anti-mouse IgG (Cappel Laboratories). The specimens were examined using a laser confocal microscope (MRC1000; Bio-Rad).

**RESULTS**

**TAP Associates with Hypophosphorylated ASF.**—We initially investigated whether the phosphorylation state of nucleocytoplasmic shuttling SR proteins affects their interaction with the mRNA export receptor TAP. HA-tagged SR protein 9G8, ASF, or SC35 was co-expressed with FLAG-tagged TAP in human 293 cells; 9G8 and ASF are shuttling proteins whereas SC35 is restricted to the nucleus (15). The cell lysates were subjected to immunoprecipitation with anti-FLAG antibody in the presence of RNase A. Detection of transiently expressed SR proteins revealed the direct interaction of FLAG-TAP with the two shuttling SR proteins (Fig. 1A, lanes 1–4), consistent with a previous report (17). In contrast, SC35 did not co-precipitate with FLAG-TAP (lanes 5 and 6). However, immunoprecipitated 9G8 and ASF showed higher electrophoretic mobility on SDS-PAGE than the corresponding lysate proteins (lanes 1–4, compare IP to input). Since ASF co-precipitated most efficiently (Fig. 1A), and its association with TAP was also observed by mass spectrometric analysis of TAP-interacting proteins (data not shown), we next examined whether the faster migrating ASF corresponds to a dephosphorylated form.
5% of the recombinant proteins used in the pull-down experiment. FLAG-tagged TAP was ectopically expressed in 293 cells and immunoprecipitated from the lysate using anti-FLAG M2-agarose. M2-agarose-bound FLAG-TAP was then incubated with recombinant GST (lane 4) or GST-ASF that was prephosphorylated with SRPK1 in the absence (lane 5) or presence (lane 6) of ATP. FLAG-TAP bound proteins were analyzed by Western blotting with anti-GST antibody. Lanes 1–3 show 5% of the recombinant proteins used in the pull-down experiment.

ASF (Fig. 1B, lanes 2 and 3) migrated slightly further than TAP-associated ASF (lane 4) and the faster migrating ASF in the mock-treated lysate (lane 1). Moreover, in vitro phosphorylation of the TAP co-precipitates using recombinant SRPK1 yielded the slower migrating ASF (Fig. 1C, lane 3). This result suggests that the RS domain within TAP-associated ASF is partially dephosphorylated and also excludes the possibility that faster migration of ASF results from proteolysis. Since phosphatase inhibitors had been included in the lysates, hypophosphorylated ASF should not be a result of dephosphorylation that occurred during the course of immunoprecipitation.

We next used recombinant ASF in pull-down experiments to examine whether its phosphorylation state influences the interaction with TAP in vitro. To obtain the TAP protein bait, the lysate of FLAG-TAP-expressing 293 cells was subjected to immunoprecipitation using anti-FLAG antibody. Immunoprecipitated FLAG-TAP was immobilized on agarose and then incubated with unphosphorylated or SRPK1-phosphorylated GST-ASF. Phosphorylated GST-ASF was assessed by its slower migration on SDS-PAGE (Fig. 2, lane 3). The pull-down data showed that only non-phosphorylated ASF bound to FLAG-TAP (lane 5), consistent with the above observation that hypophosphorylated ASF preferentially associates with TAP.

A previous report showed that the N-terminal RRM-containing domain of ASF is sufficient for its interaction with TAP (17). However, the possibility that the RS domain modulates the conformation or binding property of the RRM still remains and requires further investigation.

In vitro phosphorylation of TAP with a pool of hypophosphorylated ASF in cells. Hypophosphorylated ASF is hypophosphorylated in Triton X-100-solubilized mRNPs in vitro. A, 293 cells were fractionated into Triton X-100-soluble and -insoluble fractions. RT-PCR analysis was performed with total RNA of each fraction to detect β-actin transcripts as described (24). B, fractionation of the 293 cells that transiently expressed HA-ASF was as described for A. Immunoprecipitation was performed with antibody against RNP A1 (lanes 2, 4, 6, and 8) or anti-HA (lanes 3 and 7) antibodies; the precipitates were further treated with RNase A (lanes 4 and 8). Western blotting was performed with anti-HA. Lanes 1 and 5 show 0.1% of the fractions used for immunoprecipitation. kD, kilodalton. C, the Triton-soluble fraction was prepared from mock (lane 2) or HA-ASFexpressing (lane 3) 293 cells and then subjected to immunoprecipitation using anti-HA antibody. Total (lane 1) or co-immunoprecipitated RNAs (lanes 2 and 3) were analyzed by RT-PCR using primers specific to spliced β-actin mRNA (upper panel) or to histone H2a mRNA (lower panel). A and C, size markers are indicated in kilobases (k).

TAP Relocalizes within the Nucleus upon Overexpression of ASF—TAP localizes primarily to the nucleoplasm and is also associated with the nuclear pore complex (6). Transiently expressed FLAG-TAP distributed in a manner similar to that of endogenous TAP (Fig. 3, panels a and b). TAP interacts with several shuttling SR proteins and EJC components (2–4), most of which preferentially localize to nuclear speckles. Therefore, the majority of TAP appears not to co-localize with its interacting partners. We therefore tested whether TAP would relocate in the presence of overexpressed ASF in HeLa cells. Indirect immunofluorescence showed that co-expression of GFP-ASF causes a fraction of FLAG-TAP accumulation in nuclear speckles (Fig. 3, panels c-f). FLAG-TAP did not relocalize when GFP or GFP fused to RBM4, another splicing regulatory factor, was co-expressed (data not shown). Since hypophosphorylated SR proteins display a prominent speckle pattern (25), our observation may reflect the interaction of TAP with a pool of hypophosphorylated ASF in cells.
soluble fraction. The remaining cell debris was disrupted by sonication, and the resulting supernatant was termed the Triton-insoluble fraction. To assess the mRNP species in the subcellular fractions, we used RT-PCR analysis to examine representative β-actin transcripts (pre-mRNA and mature mRNA) in each fraction. Spliced β-actin mRNA was detected in both Triton-soluble and -insoluble factions, but intron-containing pre-mRNA was mainly present in the insoluble fraction (Fig. 4A, lanes 1–4). We therefore concluded that the Triton-soluble fraction was likely rich in both cytoplasmic and nuclear mature mRNPs, whereas the insoluble fraction contained nucleoplasmic hnRNPs, essentially consistent with a previous report (24).

The hnRNP A1 protein is associated with specific sets of hnRNP proteins and/or mRNA export factors in different mRNP complexes (24). As reported previously (24), both precursor and mature β-actin mRNAs were co-immunopurified with hnRNP A1 (data not shown). We next examined whether ASF is present in hnRNP A1-containing RNP complexes. Subcellular fractions prepared from the cells that transiently expressed HA-tagged ASF were immunoblotted with anti-HA antibody, and HA-ASF was detected in both Triton-soluble and -insoluble factions (Fig. 4B, lanes 1 and 5). Immuno precipitation of each fraction with polyclonal anti-hnRNP A1 antibodies revealed that ASF associates with hnRNP A1-containing complexes (lanes 2 and 6) in an RNA-dependent manner (lanes 4 and 8). Of particular interest was that ASF bound to hnRNP A1-containing soluble mRNPs showed higher electrophoretic mobility (compare lane 2 with lanes 1 and 3). In contrast, ASF detected in nucleoplasmic hnRNPs had a phosphorylation level similar to that of the major pool of ASF (lanes 5–7). Therefore, it is likely that cytoplasmic and nuclear diffusible mRNPs contain mainly hypophosphorylated ASF. Moreover, these results imply that hyperphosphorylated ASF is recruited to pre-mRNAs and that ASF becomes dephosphorylated upon conversion of the pre-mRNA-containing complexes into mRNPs.

We finally examined whether ASF associates with mature mRNAs in the Triton soluble fraction. Cell lysate containing HA-tagged ASF or mock lysate was subjected to immunoprecipitation using anti-HA antibody, and RNAs extracted from the immunoprecipitates were analyzed by RT-PCR. Spliced β-actin mRNA was associated with HA-ASF but was not detected in the mock precipitates (Fig. 4C, upper panel). Since SR proteins also bind intronless mRNAs (17), RT-PCR was therefore performed to examine the presence of intronless histone H2a mRNA in the immunoprecipitates containing HA-ASF. The data showed that HA-ASF associates with histone H2a mRNA in the mRNPs-enriched fraction (Fig. 4C, lower panel), suggesting that recruitment of ASF to mRNPs can also be independent of splicing.

**Discussion**

In this study, we demonstrate that hypophosphorylated ASF associates with the mRNA export receptor TAP and mature mRNPs. ASF functions in early spliceosome assembly and may act as an adaptor for mRNA export (14, 16, 17). RS domain phosphorylation likely modulates the activity of ASF in different steps during mRNA biogenesis.

SR proteins are extensively phosphorylated in their RS domain, and dynamic phosphorylation of SR proteins is integral to their function in pre-mRNA splicing. Reversible phosphorylation of SR proteins is likely important for the assembly and function of the spliceosome (14), and inappropriate phosphorylation may perturb splicing (26). Moreover, the phosphorylation level of SR proteins profoundly affects their subcellular localization (27). Importantly, phosphorylation of the RS domain is required for recruitment of SR proteins to sites undergoing transcription (28). Thus, our observation that the phosphorylation level of ASF is higher in pre-mRNA-rich hnRNPs (Fig. 4) may coincide with its co-transcriptional activity in splicing. Moreover, splicing-independent recruitment of ASF to intronless mRNAs may also occur via a transcription-coupled process. Dephosphorylation of ASF probably takes place during or at the completion of mRNA processing; however, whether this involves different pathways upon splicing dependence of ASF association with mRNA remains unclear. Since the phosphorylation level of SR proteins may affect their affinity for other proteins (14), we hypothesize that dephosphorylation of ASF initiates mRNP remodeling or triggers dissociation of mRNPs from putative nuclear anchors that attach hnRNPs in less soluble nuclear fractions for mRNP maturation (29). Moreover, hypophosphorylated ASF associates with TAP possibly for mRNA export (17) or for other RNA regulation events.

Studies with the yeast Np13p more clearly demonstrate that the phosphorylation cycle of this protein is integral to its nucleocytoplasmic transport. Np13p is phosphorylated by the cytoplasmic kinase Sky1p and is subsequently imported into the nucleus (19). Phosphorylated Np13p is recruited co-translationally to nascent mRNAs and remains bound to the mRNP (19). Recent compelling evidence indicates that phosphatase Gcl5p dephosphorylates Np13p during mRNA processing, allowing the interaction of Np13p with Mex67p (30). Thus, dephosphorylation of Np13p promotes the formation of export competent mRNPs by recruiting Mex67p. Following export, cytoplasmic phosphorylation of Np13p triggers its release from exported mRNPs, whereby directional mRNA export is achieved (30). Hence, the nuclear export of mammalian shuttling SR proteins such as ASF (this study) and 9G8β may be analogous to that of yeast Np13p. However, much remains to be learned about metazoan SR proteins. In particularly, the step(s) at which shuttling SR proteins become dephosphorylated as well as the phosphatase(s) involved awaits to be resolved. In higher eukaryotes, multiple adaptors may exist for mRNA export, such as the numerous hnRNPs, SR proteins, and the EJC; therefore, it remains an intriguing question as to whether these factors recruit TAP in a similar phosphorylation-regulated manner.

In conclusion, phosphorylation of the RS domain modulates the splicing activity of SR proteins as well as their function during mRNP maturation and/or export.

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