 Nucleic Acids Research, 2002, Vol. 30, No. 23 5301–5309

PSKH1, a novel splice factor compartment-associated serine kinase

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Received June 24, 2002; Revised and Accepted October 2, 2002

ABSTRACT

Small nuclear ribonucleoprotein particles (snRNPs) and non-snRNP splicing factors containing a serine/arginine-rich domain (SR proteins) concentrate in splicing factor compartments (SFCs) within the nucleus of interphase cells. Nuclear SFCs are considered mainly as storage sites for splicing factors, supplying splicing factors to active genes. The mechanisms controlling the interaction of the various spliceosome constituents, and the dynamic nature of the SFCs, are still poorly understood. We show here that endogenous PSKH1, a previously cloned kinase, is located in SFCs. Migration of PSKH1-FLAG into SFCs is enhanced during co-expression of T7-tagged ASF/SF2 as well as other members of the SR protein family, but not by two other non-SR nuclear proteins serving as controls. Similar to the SR protein kinase family, overexpression of PSKH1 led to reorganization of co-expressed T7-SC35 and T7-ASF/SF2 into a more diffuse nuclear pattern. This redistribution was not dependent on PSKH1 kinase activity. Different from the SR protein kinases, the SFC-associating features of PSKH1 were located within its catalytic kinase domain and within its C-terminus. Although no direct interaction was observed between PSKH1 and any of the SR proteins tested in pull-down or yeast two-hybrid assays, forced expression of PSKH1-FLAG was shown to stimulate distal splicing of an E1A minigene in HeLa cells. Moreover, a GST-ASF/SF2 fusion was not phosphorylated by PSKH1, suggesting an indirect mechanism of action on SR proteins. Our data suggest a mutual relationship between PSKH1 and SR proteins, as they are able to target PSKH1 into SFCs, while forced PSKH1 expression modulates nuclear dynamics and the function of co-expressed splicing factors.

INTRODUCTION

Pre-mRNA splicing is an essential step in the expression of most metazoan protein-coding genes. Identification of the factors involved in the process that gives rise to alternative mRNA products is a crucial question in many aspects of developmental and cell biology, including control of apoptosis (1) and tumor progression (2,3).

Splicing factors with a serine/arginine-rich domain (SR protein) and heterogeneous nuclear ribonucleoprotein (hnRNP) families are important regulators of pre-mRNA splicing (4–10). SR proteins contain at least one RNA recognition motif and an RS domain. Depending on where they bind on the pre-mRNA, SR proteins may serve as activators or repressors of splicing (11). SR proteins recruit other SR proteins to the spliceosome through RS domain interactions (12). The phosphorylation status of SR proteins may affect their function differentially in pre-mRNA recognition, spliceosome assembly and splicing catalysis (13–15). Some SR family members shuttle between the nucleus and the cytoplasm and have roles not only in nuclear pre-mRNA splicing but also in mRNA stability control (16) and, most likely, also in mRNA export (17) and other processes constituting communication between the cytoplasm and the nucleus. Components of the transcription and splicing machinery form a fine fibrogranular reticulum connecting 20–50 nuclear splice factor compartments (SFCs), subnuclear compartments enriched in snRNPs and SR proteins in various mammalian cells (18). Transcription and processing of pre-mRNA take place at active gene loci dispersed throughout the nucleoplasm. Small nuclear ribonucleoproteins, SR proteins and other RNA processing factors shuttle between these transcription sites and other locations such as the SFCs. These processes must be under strict control and may be regulated by protein phosphorylation. The nuclear SFCs respond dynamically to kinase and phosphatase inhibitors and transcriptional activity (19–21). Reversible phosphorylation of SFC components such as SR proteins may cause their release into the nucleoplasm, changing the local concentration of SR proteins available for regulating alternative splicing. Although the SR proteins were initially thought to have redundant functions, alternative splice factor/splice factor 2 (ASF/SF2) is essential for cell viability, indicating that it has at least one non-redundant function *in vivo* (22). Despite the accumulation of a considerable amount of data leading to a better understanding of the splicing machinery, our knowledge about the trafficking and targeting of proteins involved in splicing is still incomplete. A relatively limited number of kinases and phosphatases targeting components of the splicing machinery *in vivo* have been described. Protein kinases that can use SR proteins as substrates are the yet unidentified U1 70K kinase (23) and the SRPK (24) and Clk/Sty families (25), as well as DNA

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topoisomerase I (26). Additionally, dephosphorylation of SR proteins initiated by an adenovirus-encoded protein has been shown (15). PIR1, a novel member of the dual-specificity subfamily of the protein tyrosine phosphatases, may also participate in nuclear pre-mRNA metabolism (27). Many of these factors may be involved in pathways transducing extra- and intracellular signals to the splicing process (28–31). We have recently described an autophosphorylating protein serine kinase, PSKH1, which is localized to speckle-like structures in the nucleus and to the Golgi apparatus (32). This report focuses on the nuclear distribution of endogenous PSKH1 and identifies it as a SFC-associated kinase, with SR protein features. Its intracellular localization depends on the expression levels of several other members of the SR protein family, while forced PSKH1 expression antagonizes SFC targeting of co-expressed SC35 and ASF/SF2. We suggest that PSKH1 is a novel SFC-associated serine kinase with a role in intranuclear SR protein trafficking and pre-mRNA processing.

MATERIALS AND METHODS

Plasmids, cell culture and transient transfection

For transfection experiments, all plasmid DNA samples were purified using Jetstar midi-columns. Plasmid DNA, PSKH1-FLAG, the kinase-negative active site mutant $PSKH1_{D218A}$-FLAG, enhanced green fluorescent protein (EGFP)-PSKH1 and FLAG-78-360, as well as the cell culture conditions, have been described previously (32). The 1-94-EGFP fusion was constructed by PCR using the primers 5′-GTCCGGATCCG-CACCATGGGCTGTGGGACAAGC-3′ and 5′-GGACGG-ATCCTATTTGACAGGGCCAACACTGTCC-3′ and cloned into *Bam*HI-digested pEGFP-N1, while the EGFP-355-424 fusion was made using the primers 5′-GACGGATCCGTG-GTGAGCATGGCTGCCTC-3′ and 5′-CCATGGATCCCT-CAGCCATTCGGCTCAGCC-3′ and cloned into *Bam*HI-digested pEGFP-C1.

The N-terminal truncated mutant, FLAG-78-424, was constructed by PCR using the primers 5′-GTCGAAGCTTC-CACCACGCAGGGCCAGGG-3′ and 5′-CCATGGATCCC-TCAGCCATTCGGCTCAGCC-3′ and cloned into *Bam*HI/*Hin*dIII-digested pFLAG-CMV-5. Briefly, HeLa and COS-1 cells were grown in 6 cm dishes in DMEM while U2OS cells were cultured in RPMI 1640, both supplemented with 10% fetal calf serum. At the time of transfection, cells were ~60% confluent. Transfections were performed with 0.05–2 μg DNA constructs using FuGene6 according to the Roche protocol. Cells were treated with α-amanitin (30 μg/ml; Sigma) for 5 h before fixation and immunofluorescence analysis.

Immunofluorescence microscopy

Polyclonal antibodies against PSKH1 have been described previously (32). Cells were grown on coverslips in 12-well plates essentially as previously described (32). Endogenous PSKH1 was detected using a protein G-purified polyclonal anti-PSKH1 antibody (1:50 dilution) and FITC-conjugated swine anti-rabbit secondary antibody. Endogenous SC35 was detected using a monoclonal anti-SC35 antibody (1:1000 dilution) and Texas red-conjugated donkey anti-mouse secondary antibody. For single transfections, plasmids expressing T7-tagged SR proteins or FLAG-tagged PSKH1 variants were used (1.5 μl of FuGene6 and 0.5 μg DNA). For co-expression analysis, a 1:4 (0.2 and 0.8 μg) or a 4:1 (0.8 and 0.2 μg) molar ratio was used. Where necessary, pFLAG-CMV2 was used to adjust the DNA concentration to the desired level (for double transfections 1.0 μg). Twenty-four hours after transfection, cells were fixed with paraformaldehyde (4.0% in PBS) at room temperature for 15 min and then permeabilized with 0.2% Triton X-100 in PBS for 30 min or by cold methanol for 4 min at –20°C, followed by four washes in PBS. Incubation with a mouse anti-FLAG antibody [dilution 1:1000 in blocking and incubation buffer (BAI); 1% BSA, 0.1% Triton-X100 in PBS] was at 22°C in a humidifying chamber for 40 min. All incubations and washing steps were performed using BAI. After four washes, cells were incubated with the appropriate fluorochrome-linked secondary antibody at room temperature (22°C) for 30 min, rinsed four times in BAI and finally twice in PBS. In double-labeling experiments, the cells were co-incubated with an anti-PSKH1 antibody (1:100 dilution in BAI) and an anti-T7 antibody (1:2000 dilution) or the anti-PSKH1 antibody and the anti-SC35 antibody (1:300 dilution) at 22°C for 1 h. After four washes in BAI, the cells were incubated with their respective secondary antibodies, Texas red-labeled donkey anti-mouse (1:100) and FITC-labeled swine anti-rabbit (1:50), for 30 min at 22°C and washed four times in BAI and once in cold water purified in a mQ system. For co-expression of the control fusion proteins (TCF11-EGFP and MDDX28-EGFP), PSKH1-FLAG was detected using a mouse anti-FLAG antibody (1:1000) as primary and Texas red-labeled donkey anti-mouse (1:100) as the secondary antibody. Cells were counterstained with Hoechst 33258 (Sigma) to reveal the nuclear morphology and examined on a Nikon Eclipse E600 microscope with fluorescence optics and photographed through a ×40 or ×100 objective and appropriate filters. Images were captured with a cooled CCD camera (SPOT1).

E1A splicing assay

Increasing amounts of pPSKH1-FLAG or pPSKH1-D218A-FLAG DNA (0.05, 0.5 and 1.5 μg, in each case adjusted to a total of 1.5 μg DNA with pFLAG-CMV2) were co-transfected with constant amounts of the E1A minigene (pMTE1A, 0.2 μg) into HeLa or COS-1 cells. For the E1A splice assays using the mutant PSKH1 versions (pFLAG-78-360 and pFLAG-78-424) the maximum amount of DNA (1.5 μg) from the E1A assay using PSKH1-FLAG was used. RNA from two separate transfections were pooled before RT–PCR and compared with the effect on splicing from transfection of the same amount of vector DNA (1.5 μg, pFLAG-CMV2). The transfection efficiency for each DNA concentration was assessed by fluorescence microscopy as well as immunoblotting of total lysates from cells transfected with identical plasmid concentrations in parallel. RNA was isolated from similarly transfected cultures (33) after 24 h and the mRNA fraction was isolated using oligo(dT) Dynabeads (Dynal) as recommended by the manufacturer. First strand cDNA synthesis was performed with the E1A-specific primer 5′-GGTCTT-GCAGGCTCCGGTTCTGGC-3′, or the first strand was synthesized directly on the oligo(dT) beads, for 60 min at 37°C using Omniscript™ reverse transcriptase (Qiagen), or the first strand was synthesized directly simply by extending the oligo(dT) on the beads. RT–PCR was performed with a

minimum number and times of cycles (24) to maintain linearity. Denaturing, annealing and extension were at 94°C for 30 s, 62°C for 20 s and 72°C for 40 s, respectively, using the primer pair E1A-forward (5′-GTTTTCTCCTCCGAG-CCGCTCCGA-3′) and E1A-reverse (5′-CTCAGGCTCAG-GTTCAGACACAGG-3′). PCR products were resolved on a 3.0% agarose gel, stained with Syber Gold (Pharmacia) and the relative intensities of the E1A splice products were quantified directly from the gel using a Storm 840/860 PhosphorImager and ImageQuant software (Molecular Dynamics).

Immunoblot analysis, immunoprecipitation and yeast two-hybrid interaction assays

For the reciprocal protein expression analysis of T7-SC35 and PSKH1-FLAG (molar transfection ratio 4:1 or 1:4), transfected cells were lysed directly on the coverslips with SDS lysis buffer and subjected to immunoblot analysis. Proteins were resolved by 15% SDS–PAGE and blotted onto Immobilon-P transfer membranes (Millipore). The filters were blocked for 30 min with 5% skimmed milk in 1× Tris-buffered saline, followed by a 2 h incubation with a mouse monoclonal anti-T7 antibody (1:10000; Novagen), a mouse monoclonal anti-FLAG antibody (M2, 1:1000; Sigma) or a mouse monoclonal anti-β-tubulin antibody (1:5000; Boehringer Mannheim) diluted in the same blocking solution. Alkaline phosphatase-conjugated anti-mouse IgG (1:1000) was used as the secondary antibody. Detection was performed using the AP color development reagents from Amersham. Signals were quantified using a Storm 840/860 PhosphorImager. For the immunoprecipitation procedure, various pulldown protocols were followed (G. Brede, unpublished results). We here describe briefly the procedure most commonly used. Cells co-transfected with expression plasmids carrying FLAG-tagged PSKH1 and one of the T7-tagged SR proteins were cultured for 48 h and lysed in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA and 0.4% Triton X-100) for 15 min on ice. Immunoprecipitation analysis was performed using an anti-FLAG antibody (M2; Sigma) prebound to Dynabeads (Dynal) (4 µg antibody/30 µl beads). The bead/cell extract mixture was rotated at 4°C for 6 h and washed four times in HNTG buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1.0% glycerol and 0.1% Triton X-100). Co-precipitating T7-tagged SR proteins were detected by immunoblot analysis using an anti-T7 antibody (Novagen). Cell extracts and immunoprecipitates were resolved by 12% SDS–PAGE, and blotted before the filters were blocked and processed essentially as described above. Transfection efficiencies were monitored in each case by immunofluorescence analysis as well as by immunoblot analysis of the total cell extracts. Yeast two-hybrid assays were performed essentially as described (32).

RESULTS

PSKH1 localizes to splice factor compartments

PSKH1 is an autophosphorylating serine kinase belonging to the SR protein family found, among other locations, in nuclear speckles (32). This kinase has no known substrate beyond itself and its function is unknown. We hypothesized that

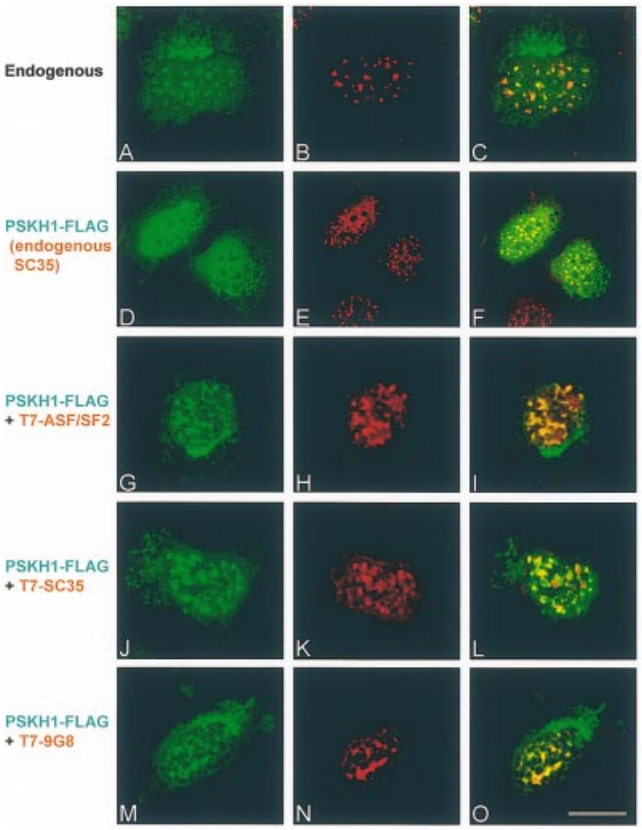

Figure 1. PSKH1 localizes to SFCs. Endogenous PSKH1 partly localizes to nuclear speckles in U2OS cells (**A**). Endogenous SC35 as a SFC marker (**B**). When superimposed, PSKH1 and SC35 at least partly co-localize to SFCs (**C**). PSKH1-FLAG (**D**). Endogenous SC35 (**E**). PSKH1-FLAG and endogenous SC35 superimposed (**F**). U2OS cells were transfected with PSKH1-FLAG (**G**, **J** and **M**) and co-transfected with a 4-fold molar excess of one of the following T7-tagged SR protein expression plasmids: T7-ASF/SF2 (**H**), T7-SC35 (**K**), T7-9G8 (**N**). (G), (J) and (M) and (H), (K) and (N) are superimposed in (**I**), (**L**) and (**O**), respectively. Endogenous PSKH1 was detected by a rabbit anti-PSKH1 antibody and a FITC-conjugated swine anti-rabbit antibody, while endogenous SC35 was detected by a monoclonal anti-SC35 antibody and a Texas red-conjugated donkey anti-mouse secondary antibody. The double transfected cells were labeled with anti-PSKH1 and anti-T7 antibodies using the same secondary antibodies as for endogenous staining. Scale bar = 10 µM.

PSKH1, being a SR-like protein and by analogy to other SR proteins, would co-localize and interfere functionally with other members of the SR protein family. We here demonstrate that endogenous PSKH1 partly co-localizes with SC35, a SFC marker and SR protein (Fig. 1A–C), although there is also a variable background of diffuse nucleoplasmic fluorescence (note that PSKH1 also localizes to the Golgi apparatus; 32). Since SR proteins are known to interact through their RS domains, we set out to analyze a possible dependence of PSKH1 localization on SR protein expression. In an attempt to understand the behavior and dynamics of endogenous PSKH1 and its relation to the SR protein family, we overexpressed various T7-tagged SR proteins and analyzed the trafficking of endogenous PSKH1. Interestingly, overexpression of T7-SC35 or T7-ASF/SF2 led to a redistribution of endogenous PSKH1 from the nucleoplasm into the SFC (data not shown).

Therefore, we also performed co-expression studies of PSKH1-FLAG with three T7-tagged members of the SR protein family (T7-ASF/SF2, T7-SC35 and T7-9G8). All three SR proteins led to a reorganization of PSKH1-FLAG from the nucleoplasm into SFCs compared to cells expressing PSKH1-FLAG only (compare Fig. 1D and E with G–I, J–L and M–O). The intranuclear localization pattern of PSKH1-FLAG was in each case highly similar to that of the overexpressed SR protein (compare Fig. 1G to H, J to K and M to N), suggesting a link between PSKH1 and members of the SR protein family. The speckle-like structures of co-expressed SR proteins were all confined to the nucleus, as revealed by staining with Hoechst 33258 (data not shown), and showed variable degrees of interconnection and loosened-up structures (compare Fig. 1G, J and M, and H, K and N). PSKH1 appeared to be excluded from promyelocytic leukemia (PML) bodies as revealed with the anti-PML antibody 5E10 (data not shown).

As a specificity control, two proteins (as EGFP fusions) known to bind nucleic acids, were tested for their effects on PSKH1 localization when overexpressed. A nuclear DNA-binding transcription factor, TCF11-EGFP, was co-expressed with PSKH1-FLAG without any effect on the intranuclear distribution of PSKH1. Likewise, co-expression of a DEAD-box helicase (MDDX28) (34) known to localize to nucleoli (and mitochondria) had no effect (data not shown).

PSKH1 targets SFCs through its kinase core and C-terminal domains

The co-expression analysis showed a marked enhancing effect of three members of the SR protein family on the SFC localization of PSKH1-FLAG, as well as on the PSKH1-EGFP and EGFP-PSKH1 fusions (data not shown). The same strategy was therefore used to identify the determinants of PSKH1 involved in its localization to SFCs. Three different PSKH1 deletion mutants were constructed. Firstly, the N-terminal domain of PSKH1 (1-94-EGFP) showed a perinuclear localization, co-staining with a Golgi marker (β-COP), similar to the wild-type PSKH1 (data not shown) and was not observed in the nucleus. Intracellular localization of 1-94-EGFP was unaffected by co-expression of either T7-ASF/SF2 or T7-SC35 (Fig. 2A–C), indicating that the N-terminal region of PSKH1 alone could not cause targeting of PSKH1 to SFCs. Secondly, the C-terminal part of PSKH1 (amino acids 355–424) was cloned C-terminal to EGFP. Although EGFP-355-424 localized diffusely to both the nuclei and the cytoplasm, in most cells EGFP-355-424 accumulated in the nuclei, sometimes with an enhanced presence at nucleolar structures (Fig. 2L, arrows and phase contrast inset) in interphase cells. No significant SFC pattern was observed in U2OS cells with the EGFP-355-424 fusion expressed alone and no significant change in the localization of this fusion was observed when co-expressed with T7-ASF/SF2 (Fig. 2G–I). Given that the C-terminal part of PSKH1 has the highest similarity to the SC35 splice factor, T7-SC35 was co-expressed with EGFP-355-424 to test possible dependence of distribution. To a certain extent, EGFP-355-424 migrated into T7-SC35-positive SFCs (Fig. 2J–L), suggesting that the C-terminus of PSKH1 alone harbors some SFC targeting features, with putative specificity for SC35. Finally, a double truncated PSKH1 version, after deletion of amino acids 1–77 and 361–424, leaving the catalytic kinase domain (FLAG-78-360), was expressed. Most of the cells expressing this double deletion mutant showed nuclear accumulation with a (diffuse) nuclear distribution and total exclusion from the perinuclear region. In some cells, the deletion mutant also associated with nuclear speckles, which to a large extent overlapped with endogenous SC35 speckles (data not shown). More importantly, when overexpressing T7-ASF/SF2 (or T7-SC35 or T7-9G8), the catalytic kinase domain strongly associated with SFCs (Fig. 2D–F). Unlike the SR kinases, which target their substrates through RS domains, we conclude that PSKH1 may associate with SFCs through its catalytic core domain.

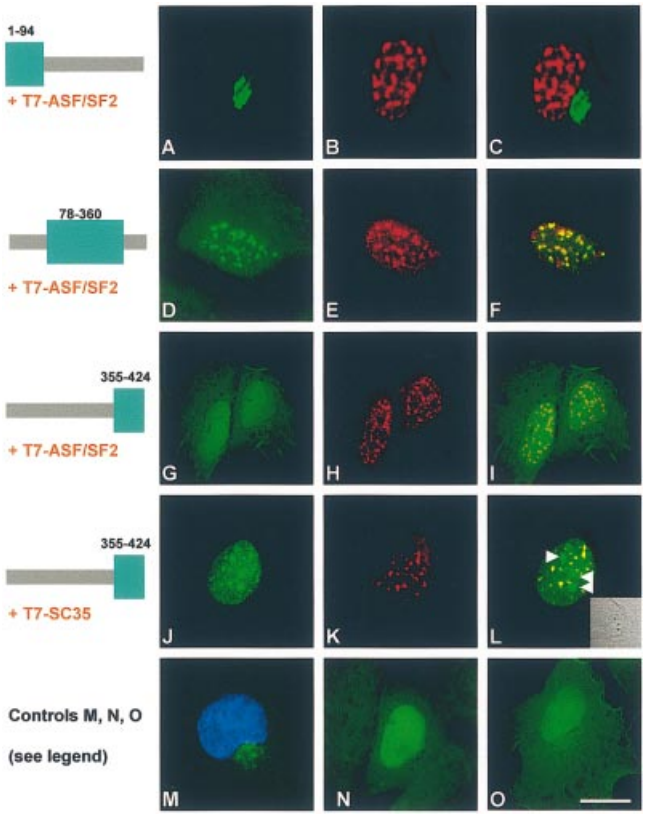

Figure 2. PSKH1 targets SFCs through its kinase core and C-terminal domains. Three PSKH1 deletion mutants: Δ95-424 (1-94-EGFP), Δ1-77, Δ361-424 (FLAG-78-360) and Δ1-354 (355-424-EGFP) were separately co-transfected into U2OS cells with a 4-fold molar excess of T7-ASF/SF2 or T7-SC35 expression plasmid. 1-94-EGFP in cells co-expressing T7-ASF/SF2 (**A**). Same cell as in (A) visualizing T7-ASF/SF2 (**B**). (A) and (B) are superimposed in (**C**). The catalytic kinase domain (FLAG-78-360) migrates into SFCs when T7-ASF/SF2 is co-expressed (**D**). T7-ASF/SF2 speckles during FLAG-78-360 co-expression (**E**). (D) and (E) are superimposed in (**F**). The C-terminal EGFP-355-424 fusion (**G**) shows no significant increase in SFCs when co-expressed with T7-ASF/SF2 (**H**). EGFP-355-424 and T7-ASF/SF2 are superimposed in (**I**). EGFP-355-424 migrates into SFCs when co-expressed with T7-SC35 (**J**). T7-SC35 in cells co-expressing EGFP-355-424 (**K**). EGFP-355-424 and T7-SC35 are superimposed in (**L**). A reduced size frame (phase contrast) of the same cell demonstrates the dense nucleolus structures (L, inset; arrowheads indicate increased staining of nucleolar structures). Control of the intracellular localization pattern of 1-94-EGFP without co-expressing T7-ASF/SF2 (**M**) (nucleus stained blue), FLAG-78-360 without co-expressing T7-ASF/SF2 (**N**) and EGFP-355-424 without co-expressing T7-ASF/SF2 (**O**). Scale bar = 10 μM.

Forced PSKH1 expression antagonizes SFC targeting of co-expressed SC35 or ASF/SF2

Phosphorylation within the RS domains of SR proteins controls intracellular and subnuclear distribution of these proteins in interphase cells as well as reorganization of SFCs during mitosis. Furthermore, it is believed that phosphorylation of SR proteins is a prerequisite for release from SFCs and participation in co-transcriptional splicing (24,25,35). Kinases present in SFCs therefore potentially may be involved in optimization of splicing factor concentration, recycling, regulation of splicing factor ratios or assembly of the transcription/processing machinery (18). Given the above results, we hypothesized that PSKH1 is somehow involved in processes within SFCs and initially looked for eventual changes in SFCs during forced expression of full-length PSKH1. This analysis showed that cells transiently co-expressing T7-ASF/SF2 and PSKH1-FLAG display a 'loosened up' speckled pattern (Fig. 3B, panels A–C, small arrowheads) when stained for ASF/SF2, compared to cells expressing T7-ASF/SF2 alone (Fig. 3B, panels B and C, large arrowheads), which showed a normal SFC pattern. To test whether this effect was specific for ASF/SF2 or was true for other SR proteins, the impact of transiently overexpressed PSKH1-FLAG (Fig. 3B, panels D–F, small arrowheads) on co-expressed T7-SC35 was tested. Interestingly, >70% of the cells (100 cells analyzed) overexpressing PSKH1-FLAG while co-expressing T7-SC35 showed essentially no speckle-like organization of T7-SC35 compared to cells expressing T7-SC35 alone, which exhibited a normal SFC pattern (Fig. 3B, panels E and F, large arrowheads). PSKH1 kinase activity was not required for this to occur since the same effect on co-transfected T7-SC35 was observed using a kinase-negative mutant ($PSKH1_{D218A}$-FLAG; Fig. 3B, panels G–I, large and small arrowheads, respectively).

The expression levels of the co-transfected PSKH1 and SR protein expression plasmids used in the immunofluorescence experiments were monitored by SDS–PAGE and immunoblot analysis. They were detected simultaneously on the same immunoblot using anti-FLAG and anti-T7 antibodies to detect PSKH1 and the SR proteins, respectively. The relative protein expression levels were consistent with the plasmid ratios used in the transfection (examples of PSKH1-FLAG and T7-SC35 expression levels are shown in Fig. 3B).

We note that forced PSKH1-FLAG expression influenced the subcellular localization of endogenous SC35 (Fig. 1D–F and data not shown) in <20% of the transfected cells (transfection efficiencies typically between 20 and 30%), suggesting that the effect observed may somehow be dependent on co-expression.

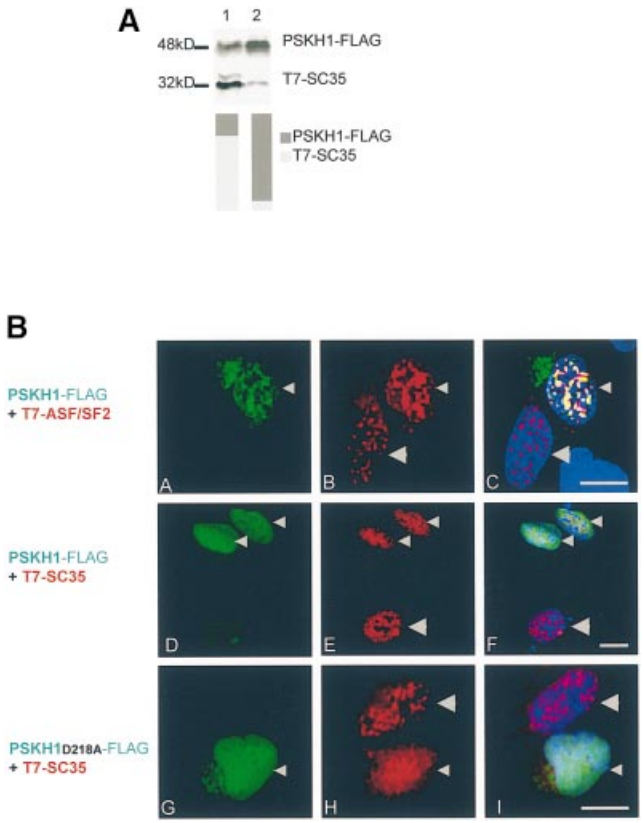

Figure 3. (**A**) Protein expression level of PSKH1-FLAG and T7-SC35 in co-transfection assays. Immunoblot analysis of cells co-transfected with a 4-fold molar excess of T7-SC35 relative to PSKH1-FLAG (lane 1) and a 4-fold molar excess of PSKH1-FLAG relative to T7-SC35 (lane 2). The relative level of each co-expressed tagged protein is shown at the bottom of the figure. (**B**) Transient overexpression of PSKH1-FLAG leads to a redistribution of co-expressed T7-SC35 and T7-ASF/SF2. U2OS cells were co-transfected with PSKH1-FLAG (A–F) or its kinase-negative mutant (G–I) and T7-ASF/SF2 (A–C) or T7-SC35 (D–I). A 4-fold molar excess of PSKH1 coding plasmid (or its corresponding mutant DNA) compared to SR coding plasmid was applied. Forced expression of PSKH1-FLAG (or mutant) antagonizes the normal speckled appearance of SC35 (E, F, H and I, small arrowheads) in more than 70% of the double transfected cells. Cells not expressing PSKH1 (or mutant) (large arrowheads) all show a normal speckled pattern. The localization of PSKH1-FLAG in the Golgi apparatus was often strongly reduced or absent in cells co-expressing T7-tagged SR protein (D and F). Scale bar = 10 μM.

Redistribution of PSKH1 and SC35 during transcriptional arrest

Increasing evidence suggests that the pre-mRNA splicing machinery is influenced by components of the transcriptional apparatus. Involvement of the C-terminal domain of RNA polymerase II at multiple steps in pre-mRNA processing, including splicing, has been demonstrated (36). Having established a relationship between PSKH1 and members of the SR protein family, we asked whether the subcellular localization of endogenous PSKH1 was dependent on ongoing transcription. U2OS cells were treated with α-amanitin for 5 h before immunofluorescence analysis. Endogenous PSKH1 was distributed into enlarged, rounded-up nuclear speckles co-localizing with SC35 (Fig. 4A–C). The effect of transcription inhibition on localization of an EGFP-PSKH1 fusion was also tested. This fusion has lost its Golgi targeting potential and showed behavior similar to the wild-type with respect to SR protein dependence and nuclear distribution. As with the effect on endogenous PSKH1, transcription inhibition by α-amanitin resulted in migration of EGFP-PSKH1 from a diffuse nuclear pattern (data not shown) into 5–10 enlarged and rounded-up nuclear speckles positive for SC35 (Fig. 4D–F). Similar results were obtained with a different transcriptional inhibitor, 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB), in HeLa and COS-1 cells (data not shown). EGFP alone did not change its nuclear distribution

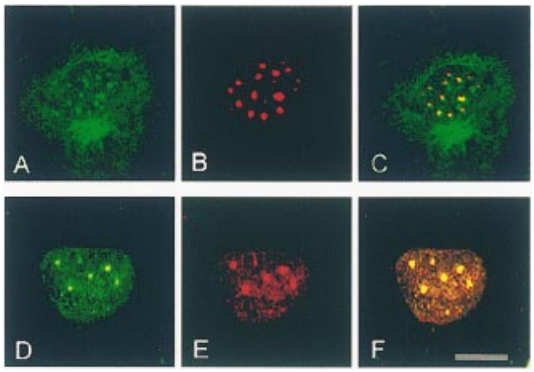

Figure 4. Nuclear reorganization of PSKH1 during transcriptional arrest. Endogenous PSKH1 (**A**) or transfected EGFP-PSKH1 fusion (**D**) reorganizes into enlarged and rounded-up speckles together with endogenous SC35 (**B** and **E**) upon transcriptional arrest (**C** and **F**). Scale bar = 10 μM.

upon transcriptional arrest (data not shown). PSKH1 and SC35 thus show similar intranuclear rearrangement under transcriptional arrest.

PSKH1 overexpression affects splicing of an E1A minigene

The ability of PSKH1 to associate with SFCs and also, during overexpression, to antagonize the presence of co-expressed T7-SC35 (and T7-ASF/SF2) in SFCs might result in changes in the concentration of locally available ASF/SF2 and SC35 proteins. A high concentration of ASF/SF2 activates proximal cryptic splice sites (6). The effect of full-length PSKH1 (PSKH1-FLAG) on alternative splicing of an E1A minigene (pMTE1A) was therefore tested. Three possible 5′ splice sites used by the virus during infection result in adenovirus E1A mRNA species of 9S, 12S and 13S (37). The E1A splicing analysis indicated that forced expression of PSKH1-FLAG resulted in activation of the distal 9S splice site in a dose-dependent manner, manifested as an increased level of 9S mRNA compared to the vector control (Fig. 5, lanes 2–5). Given that the effect on co-expressed T7-SC35 or T7-ASF/SF2 during forced expression of PSKH1 was independent of the kinase activity of PSKH1, an identical assay was performed using the kinase-negative $PSKH1_{D218A}$-FLAG. Although not to the same extent as PSKH1-FLAG, $PSKH1_{D218A}$-FLAG also seemed to alter E1A splicing in a dose-dependent manner by increasing the level of 9S splice product, suggesting that the effect on splicing is at least partly independent of kinase activity. This is also in agreement with the co-expression experiments with T7-SC35 and $PSKH1_{D218A}$-FLAG (Fig. 3B, panels G–I). Importantly, the (nuclear targeting only) N-terminal deleted mutant (FLAG-78–424) stimulated formation of the 9S splice product more potently (Fig. 5D, lane 4) than full-length PSKH1-FLAG, while the double truncated mutant (FLAG-78-360) showed no significant effect on splicing (Fig. 5D, lane 2), suggesting a crucial role of the C-terminal domain of PSKH1. This is in agreement with the immunofluorescence results showing that the SFC targeting feature of the EGFP-355-424 C-terminal fusion peptide is enhanced when co-expressing T7-SC35 but not T7-ASF/SF2 (Fig. 2I–O). Forced T7-ASF/SF2 expression resulted in elevated levels of the 13S proximal splice product as expected, with the 9S and 12S RNA species being essentially absent (Fig. 5B, lane 1, and D, lane 3). For PSKH1-FLAG and the truncated mutants (FLAG-78-360 and FLAG-78-424) three independent experiments were performed, while for $PSKH1_{D218A}$-FLAG two independent experiments were performed. Relative ratios of 13S, 12S and 9S E1A mRNA were determined and are shown schematically.

DISCUSSION

Given the high frequency of genes with varying patterns of alternative splicing of their pre-mRNA and that ~15% of all human genetic diseases are caused by mutations destroying functional splice sites or generating new ones (2), it is important to identify the molecules involved in these processes. SR family proteins and SR protein kinases are involved in such pathways by functioning as splice factors or splice factor activators. The present paper describes for the first time a link between PSKH1 and members of the SR protein family involved in pre-mRNA splicing. Endogenous as well as transiently overexpressed PSKH1 associated with SFCs, and increasingly so during co-expression (4-fold excess DNA) of ASF/SF2 or other members of the SR family. T7-tagged SC35 or ASF/SF2, on the other hand, relocated from SFCs to a more diffuse nuclear pattern during forced expression of PSKH1 (4-fold excess), but not when co-transfected with two nuclear control proteins (data not shown). Thus our data indicate a role of PSKH1 in regulating the intranuclear distribution or level of SC35 and/or other members of the SR protein family in SFCs.

PSKH1 seems not to work through direct SR protein phosphorylation

Like the SRPK family (38), PSKH1 is found both in the cytoplasm and in the nucleus, whereas Clk/Sty kinases appear to be exclusively located in the nucleus (25,39). Phosphorylation of the RS domain directs subcellular localization of SR proteins (19,34) and overexpression of the Clk/Sty kinase results in a diffuse distribution of SR proteins (26). However, unlike the SRPK and Clk/Sty families of kinases, PSKH1 also associated with the Golgi apparatus, suggesting a unique feature of PSKH1. No substrate has been identified for PSKH1 (except itself), suggesting that PSKH1 does not act directly on SR proteins through phosphorylation.

Analysis of the phopshorylation status of the SR proteins in extracts from cells overexpressing PSKH1 or various PSKH1 mutants was attempted using the monoclonal antibody mAb104 (which recognizes the phospho-SR epitope). The difficulty in detecting a change in the phosphorylation status of the SR proteins is consistent with the observation that the kinase-negative mutant ($PSKH1_{D218A}$-FLAG) was also able to displace ASF/SF2 and SC35 from speckles when co-expressed. These observations are also in line with the fact that PSKH1 is unable to use a bacterially expressed and purified GST-ASF/SF2 fusion as substrate (data not shown). Although both the SRPK family and the Clk family can use ASF/SF2 as substrate *in vivo* (which PSKH1 apparently cannot), our results suggest that PSKH1 is able to induce similar net effects on the intranuclear structural organization of SC35 and ASF/SF2 when co-expressed. Different from the

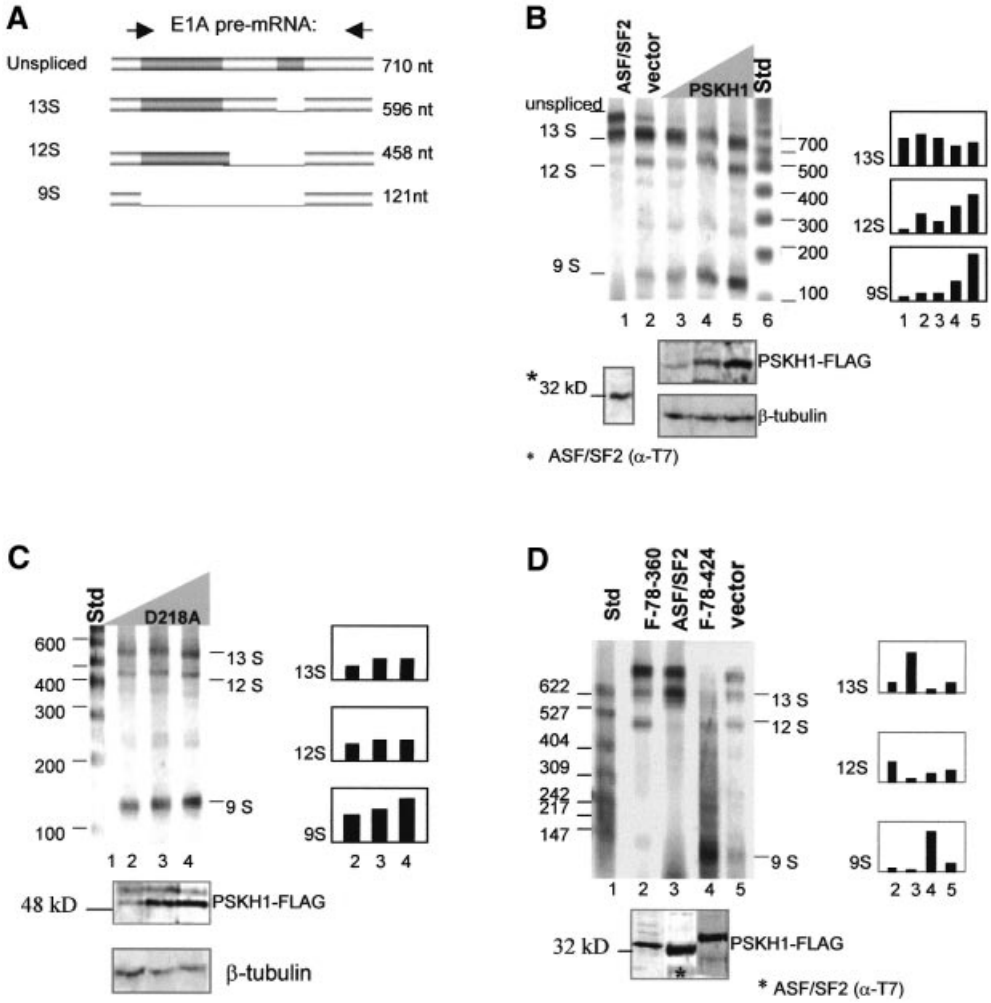

Figure 5. PSKH1 affects splicing of adenovirus E1A pre-mRNA in HeLa cells. (**A**) Schematic representation of the E1A minigene, showing various alternative splice sites, the positions of the primers used for RT–PCR and the expected lengths of the resulting RT–PCR products. (**B**) Lane 1, mRNA expressed from the plasmid pMTE1A co-transfected with pT7-ASF/SF2; lane 2, plasmid pMTE1A co-transfected with the vector control; lanes 3–5, plasmid pMTE1A co-transfected with increasing amounts of pPSKH1-FLAG (0.05, 0.5 and 1.5 μg); lane 6, MassRuler™ DNA ladder, low range (Fermentas). Each bar represents the average of three experiments and shows schematically the level of each splice product. As controls for PSKH1-FLAG protein expression, identical plasmid combinations as used in the RT–PCR analysis were run in parallel and subjected to immunoblot analysis. PSKH1-FLAG was detected using a monoclonal anti-FLAG antibody (M2). As a control for equal loading, β-tubulin was monitored with an anti-β-tubulin antibody. Protein expression level control for T7-ASF/SF2 was performed using the anti-T7 antibody. (**C**) Lane 1, MassRuler™ DNA ladder, low range (Fermentas); lanes 2–4, plasmid pMTE1A co-transfected with increasing amounts of pPSKH1-D218A-FLAG (0.05, 0.5 and 1.5 μg). Each bar represents the average of two experiments. As a control for $PSKH1_{D218A}$-FLAG protein expression, parallels with identical plasmid combinations (as used in the RT–PCR assay for lanes 2–4) were transfected into cells and subjected to immunoblot analysis. β-Tubulin was used as a control for equal loading. (**D**) Lane 1, *Msp*I-digested pBR322; lane 2, plasmid pMTE1A co-transfected with pFLAG-78-360 (1.5 μg); lane 3, plasmid pMTE1A co-transfected with pT7-ASF/SF2 (0.5 μg); lane 4, plasmid pMTE1A co-transfected with pFLAG-78-424 (1.5 μg); lane 5, plasmid pMTE1A co-transfected with vector control (1.5 μg). The results in (D) are representative of at least three separate experiments for each expression plasmid. The positions of the 13S, 12S and 9S transcripts are indicated at the side of each gel. Gels were stained with Cyber Gold. RNA was harvested 24 h after transfection and analyzed by RT–PCR. DNA standard, *Msp*I-digested pBR322.

Clk and SRPK kinase families, however, PSKH1 seemed not to be directly physically associated with the SR proteins. This was tested in cell extracts made from transfected cells by performing pull-down assays (with both full-length PSKH1-FLAG, GST-PSKH1, FLAG-78-424 and FLAG-78-360) and in yeast two-hybrid assays. None of the SR proteins SC35, ASF/SF2, U2AF65, U2AF35, SRp20, UI70K, p80-coilin, SRp30c, SRp40, R6M and 9G8 interacted with PSKH1 in yeast two-hybrid interaction assays. However, the kinase activation mechanism of PSKH1 is unknown and the possibility of additional activating factors being required for SR protein phosphorylation or other interactions cannot be excluded. Thus, our results suggest that the reciprocal effect on PSKH1 and SR proteins is mediated through indirect mechanisms, independent of kinase activity but possibly dependent on a (unstable) bridging factor, such as RNA.

PSKH1 interacts with SFCs through its catalytic kinase core domain as well as its SR-rich C-terminus

The SR-rich C-terminal domain of PSKH1 (EGFP-355-424, containing six SR/RS dipeptides) targeted SR speckles in co-expression assays. While EGFP-355-424 did not apparently increase its distribution to SFCs when T7-ASF/SF2 was co-expressed, EGFP-355-424 targeted strongly to SFCs when

T7-SC35 was co-expressed. The same region (355–424) shows highest similarity to the SR region of SC35 (data not shown) and the redistribution effect on SR proteins during forced PSKH1-FLAG expression was more dramatic for T7-SC35 than for T7-ASF/SF2 (Fig. 3A, compare panel B with E and H). Although too few SR proteins were tested, PSKH1 may exhibit some preference for SC35, at least compared to ASF/SF2. Due to the presence of only one SR dipeptide within FLAG-78-360 (at wild-type positions 110–111), it was unexpected that the catalytic kinase domain of PSKH1 alone associated with SFCs during co-expression of T7-ASF/SF2 or T7-SC35. PSKH1 thus has the potential to interact with factors of the RNA processing machinery through its conserved kinase domain, to our knowledge a novel feature of kinases involved in RNA processing. We cannot exclude the possibility that the kinase domain associates with SFCs indirectly by dimerization or oligomerization. Any such factor would not appear to be limiting, since overexpression of FLAG-78-360 led to an increased presence of the mutant protein in SFCs. The effect of the doubly truncated (FLAG-78-360) as well as the N-terminal deletion mutant (EGFP-355-424) may suggest that SR-dependent as well as SR-independent structures are involved in these interactions.

PSKH1 seemed to be present in SFCs as well as diffusely in the nucleoplasm in transcriptionally active nuclei. However, in the presence of the transcription inhibitor α-amanitin (and DRB), endogenous PSKH1, as well as EGFP-PSKH1, appeared to be associated with enlarged and rounded-up nuclear SFCs, co-localizing with SC35. PSKH1 therefore behaved in a similar manner to members of the SR protein family during transcriptional arrest (40), consistent with a role for PSKH1 in SR protein subnuclear organization and/or RNA processing.

Effect of PSKH1 in an *in vivo* E1A splicing assay

Forced PSKH1 expression affected the splicing pattern of the E1A minigene in a concentration-dependent fashion. The *in vivo* splicing effect of $PSKH1_{D218A}$–FLAG resulted in a slightly increased level of 9S RNA, in line with the similar effect of co-expressing the D218A mutant or wild-type PSKH1 with T7–SC35 (compare Fig. 3B, panels D–F and G–I). Although our results point in the direction of general splice activation, they also displayed differentially enhanced 9S splicing in HeLa cells. PSKH1 therefore behaved in a similar way to the hnRNP A/B group of proteins, which activate the most distal 5′ splice site of adenovirus E1A pre-mRNA. We observed a more potent effect on splicing using the N-terminal mutant (FLAG-78-424), compared to the wild-type. This could simply be explained by the increased nuclear localization of this mutant. However, the double truncated mutant (FLAG-78-360) also exhibited a similar nuclear presence and expression level without significant effect on splicing, suggesting a role for the C-terminal region of PSKH1 in mediating the effect on splicing. This is also in line with our result demonstrating that the C-terminus alone could target SFCs when T7-SC35 was co-expressed. The molecular explanation behind this effect remains to be determined. PSKH1 may alter E1A splicing through its effect on the intranuclear organization of members of the SR protein family, thereby changing the local concentration and/or the phosphorylation status of the available essential splice factors such as ASF/SF2 and/or SC35. Alternatively, PSKH1 may affect E1A splicing through other as yet unknown mechanisms.

Overproduction of proteins may cause cellular stress. The expression of two nucleic acid-binding proteins (MDDX28 and TCF11) did not, however, cause any obvious changes similar to those caused by forced expression of PSKH1, arguing against the hypothesis that migration of PSKH1 into SFCs is a result solely of the stress of high transcription activity or protein expression level.

Conclusions

PSKH1 has several features suggesting that this protein serine kinase has a role in SR protein nuclear distribution and thus in RNA processing. Endogenous PSKH1 localizes to nuclear speckles overlapping with SFCs and is concentrated in SFCs during overexpression of SR proteins such as SC35 and ASF/SF2. To our knowledge this is the first example where SR protein overexpression drives a kinase into SFCs. The minimal determinants able to associate it with SFCs are harbored within the catalytic kinase domain. A C-terminal domain (355–424) is able to target SFCs during T7-SC35 co-expression, suggesting an additional SFC targeting role for the SR-rich C-terminal domain. Moreover, forced expression of full-length PSKH1 induces release of co-expressed SC35 from SFCs. PSKH1 migrates into enlarged, rounded-up nuclear SFCs along with SC35 during transcriptional arrest. Furthermore, PSKH1 affects splicing by stimulating distal splicing of an E1A minigene in a concentration-dependent manner. Our data suggest that PSKH1 is a novel actor in the control of the trafficking of SR proteins with subsequent effect on pre-mRNA processing.

ACKNOWLEDGEMENTS

We are grateful to Dr A. Krainer and Lisa Manche for providing us with the pMTE1A plasmid, Dr J. F. Caceres and Prof. N. Hastie for the T7-tagged and two-hybrid SR protein plasmids, Dr Cathrine Husberg and Prof. A. B. Kolsto for the TCF11-ECFP plasmid and Dr K.-H. Kalland for the anti-SC35 antibody. This work was supported by grants to H.P. from the Research Council of Norway, the Norwegian Cancer Society and the Jahre Foundation. G.B was a Research Fellow of the Norwegian Cancer Society and of the University of Oslo.

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