Differential effects of hyperphosphorylation on splicing factor SRp55

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Members of the serine/arginine-rich (SR) protein family play an important role in both constitutive and regulated splicing of precursor mRNAs. Phosphorylation of the arginine/serine dipeptide-rich domain (RS domain) can modulate the activity and the subcellular localization of SR proteins. However, whether the SR protein family members are individually regulated and how this is achieved remain unclear. In this report we show that 5,6-dichloro-1 β -D-ribofuranosyl-benzimidazole (DRB), an inhibitor of RNA polymerase II-dependent transcription, specifically induced hyperphosphorylation of SRp55 but not that of any other SR proteins tested. Hyperphosphorylation of SRp55 occurs at the RS domain and appears to require the RNA-binding activity. Upon DRB treatment, hyperphosphorylated SRp55 relocates to

enlarged nuclear speckles. Intriguingly, SRp55 is specifically targeted for degradation by the proteasome upon overexpression of the SR protein kinase Clk/Sty. Although a destabilization signal is mapped within the C-terminal 43-amino acid segment of SRp55, its adjacent lysine/serine-rich RS domain is nevertheless critical for the Clk/Sty-mediated degradation. We report for the first time that SRp55 can be hyperphosphorylated under different circumstances whereby its fate is differentially influenced.

Key words: nuclear speckles, protein degradation, serine/arginine-rich (SR) protein kinase, transcription inhibitor.

INTRODUCTION

The removal of introns from eukaryotic precursor mRNAs is a critical aspect of gene expression. Moreover, through alternative pre-mRNA splicing pathways, a single gene can generate functionally diverse protein polypeptides, thereby increasing genome complexity [1]. Members of the serine/arginine-rich (SR) protein family play an important role in both constitutive and regulated pre-mRNA splicing [2–4]. Some of SR proteins are also involved in mRNA export and stability regulation [5,6]. All SR proteins exhibit a modular organization in their structure [2,3]. A set of prototypical SR proteins contains one or two RNA-recognition motifs (RRMs) at the N-terminus and an arginine/serine dipeptide-rich domain (RS domain) consisting of multiple repeats of arginine/serine dipeptides at the C-terminus [2,3]. The RRM bind to RNA and can determine substrate specificity whereas the RS domain mediates protein–protein interactions between SR proteins [2,3]. Through the interactions with RNA *cis*-elements or with other splicing factors, SR proteins execute their constitutive functions in pre-mRNA splicing and regulate splice-site selection in pre-mRNAs that undergo alternative splicing [2,3].

SR proteins *in vivo* are extensively phosphorylated in their RS domain [7]. Numerous lines of evidence have indicated that the phosphorylation status of the RS domain can influence the RNA binding specificity, splicing activity and subcellular localization of SR proteins, and also affect as well the interactions between SR proteins [8–17]. Earlier observations have shown that reversible phosphorylation of splicing factors including SR proteins is required for assembly and function of the active spliceosome [11,12]. However, both hyper- and hypophosphorylation of the RS domain inhibit the splicing activities of SR proteins [13]. Therefore, appropriate and dynamic phosphorylation is crucial

to the splicing reaction. Phosphorylation may improve the RNA binding specificity of SR proteins by reducing the non-specific interactions between hypophosphorylated RS domain and RNA [8]. Moreover, the interactions between SR proteins and other spliceosomal constituents can be differentially affected by RS domain phosphorylation/dephosphorylation [10]. SR proteins are predominantly located in nuclear speckles and redistribute to the nucleoplasm or to the cytoplasm while hyperphosphorylated [14–16]. In contrast, incubation of permeabilized cells with protein phosphatase (PP) 1 enlarges nuclear speckles [17]. Thus phosphorylation is implicated in the release of SR proteins from the speckles to the sites of active transcription where they function in pre-mRNA splicing [18,19].

Several types of mammalian protein kinases including the SR protein kinase (SRPK) and Clk/Sty families can phosphorylate SR proteins [15,16,20]. The Clk/Sty kinases are predominantly localized in nuclear speckles whereas SRPKs exist in both the nucleus and the cytoplasm [20,21]. These two families of kinases appear to have distinct but overlapping substrate specificities [22]. So far, it is not yet clear whether they play redundant or completely different roles in regulating SR splicing factors. Reversible phosphorylation of SR proteins involves protein phosphatases. Inhibition of PP1 and PP2A activities blocks the catalytic steps of splicing without affecting spliceosome assembly [11,23]. SR proteins and U1 small ribonucleoprotein-specific 70 kDa protein appear to be the targets of these phosphatases [23]. PP2C γ is physically associated with the spliceosome, and its phosphatase activity is required for an early step in spliceosome assembly [24]. Thus dynamic phosphorylation of SR proteins is integral to their functions in pre-mRNA splicing and warrants further investigation.

How cellular signalling modulates the activity or phosphorylation level of SR proteins *in vivo* remains a tantalizing puzzle.

Abbreviations used: DRB, 5,6-dichloro-1 β -D-ribofuranosyl-benzimidazole; HA, haemagglutinin; RRM, RNA-recognition motif; RS domain, arginine/serine dipeptide-rich domain; SR, serine/arginine-rich; SRPK, SR protein kinase; PP, protein phosphatase; NP-40, Nonidet P-40; mAb, monoclonal antibody; ASF, alternative splicing factor; SC35, splicing component 35 kDa; MDCK cells, Madin–Darby canine kidney cells.

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The kinase activity of SRPK1 increases during mitosis and SR proteins thus become hyperphosphorylated [15]. Recent reports have shown that ceramide treatment of the cells induces dephosphorylation of SR proteins via activation of FAS [25]. Dephosphorylation of SR proteins may enhance the execution of the apoptotic response through modulation of alternative splicing of caspase 9 and Bcl-x [26]. During mitosis SRp38 becomes dephosphorylated and acts as a general splicing repressor, indicating that mitotic gene silencing can be regulated by dephosphorylation of a specific SR protein [27]. Moreover, the phosphorylation level of SR proteins can be modulated upon adenovirus infection and during the development of the nematode embryo [28,29]. However, how the cellular signals transmit to SRPKs or phosphatases is currently unknown.

In this report, we show that SRp55 behaves differently from other SR proteins when assessed by the inhibition of transcription by 5,6-dichloro-1 β -D-ribofuranosyl-benzimidazole (DRB) or overexpression of the Clk/Sty kinase. Interestingly, the fate of SRp55 is influenced differentially by hyperphosphorylation via different pathways.

EXPERIMENTAL

Plasmids

The mammalian expression vectors of wild-type and mutant Clk/Sty were obtained from Dr J. Bell (University of Ottawa, Ottawa, Canada) [16]. The SRp55 and SRp75 cDNAs were a generous gift from Dr A. Krainer (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A.) [30], whereas the cDNAs of alternative splicing factor (ASF), splicing component of 35 kDa (SC35) and SRp40 were obtained by reverse-transcriptase PCR using total RNA prepared from HeLa cells as a template. These cDNAs were then inserted into pCEP4 (Invitrogen) in frame with the C-terminal haemagglutinin (HA) epitope tag. Analogously, DNA fragments encoding SRp55 deletion mutants Δ C21 (deletion of amino acids 324–344) and Δ C43 (deletion of 302–344) were generated by PCR and inserted into HA-tag-containing pCEP4. The expression vectors for chimaeric SR proteins were as follows: RRM⁴⁰-RS⁵⁵, which is SRp40 RRM (amino acids 1–175) fused to the SRp55 RS domain (amino acids 182–344); RRM⁵⁵-RS⁴⁰, the SRp55 RRM (amino acids 1–181) fused to the SRp40 RS domain (amino acids 176–272); ASF-C43, the SRp55 C-terminal 43 amino acids C-terminally fused to full-length ASF; and RRM^{ASF}-RS⁵⁵, the ASF RRM (amino acids 1–185) fused to the SRp55 RS domain (amino acids 182–344). All the SRp55 deletion mutants and SR protein chimaeras contained in-frame HA tags at the C-terminus, and their sequences were verified by automatic sequencing.

Cell culture, transfection and establishment of stable cell lines

HeLa, HEK-293, COS-1, NIH3T3 and Madin–Darby canine kidney (MDCK) cells were grown in Dulbecco's modified Eagles' medium supplemented with 10 % fetal bovine serum and L-glutamine. Transfection using LipofectAMINE (Invitrogen) was performed as described previously [31]. To detect transiently expressed proteins, cells were harvested 44 h post-transfection and the cell lysate was prepared (see below). To select cells that ectopically express exogenous SR proteins, cells were transfected as above and subsequently cultured in the medium containing 200 μ g/ml hygromycin (Clontech) for 2 weeks. Resistant colonies were pooled and subjected to DRB treatment and lysate preparation (see below). To establish the cell lines stably

expressing HA-tagged SRp55, HeLa cells were transfected with pCEP4-SRp55.HA and cultured under selection with 200 μ g/ml hygromycin for 2 weeks. Resistant colonies were picked and seeded in 24-well culture dishes, and selection continued for an additional 2 weeks. Finally, the surviving cells were screened for stable expression of SRp55.HA using Western blotting.

Drug treatment

Compounds used to inhibit transcription included DRB (100 μ M; Sigma), actinomycin D (10 μ g/ml; Sigma) and H-7 (100 μ M; Calbiochem). Post-transfection (20 h) cells were trypsinized, aliquoted equally into two dishes and cultured for a further 20 h followed by drug or mock treatment for 3 h. To test the effect of the proteasome inhibitor on SRp55 stability, HeLa cells were co-transfected with vectors expressing HA-tagged SRp55 and Clk/Sty or its mutant for 8 h. Then 10 μ M lactacystin (Calbiochem) was added to the culture medium 15 h prior to cell harvest.

Preparation and fractionation of the cell lysate

Cells were collected by centrifugation and the cell pellet was resuspended in buffer A containing 10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 % Nonidet P-40 (NP-40) and protease inhibitor cocktail (Roche Molecular Biochemicals). Resuspended cells were incubated on ice for 5 min. After centrifugation, the pellet was washed with cold PBS and the supernatant analysed directly if necessary. Nuclear proteins were finally extracted from the pellet with SDS sample buffer and subjected to Western blot analysis.

Phosphatase treatment

To prepare nuclear extracts for phosphatase treatment, the nuclei were resuspended in RIPA buffer containing 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1 % NP-40, 1 mM dithiothreitol, 1 mM PMSF, 1.5 μ g/ml aprotinin and 2 μ g/ml leupeptin for 30 min on ice. Nuclear extract (6 μ l) was incubated with 10 units of calf intestine alkaline phosphatase (Roche Molecular Biochemicals) in a 10 μ l reaction containing 50 mM Tris/HCl (pH 8.5), 1 mM EDTA and protease inhibitors for 30 min at 37 °C.

Western blotting

Proteins were fractionated on SDS/polyacrylamide gels (10 %) and transferred on to nitrocellulose membranes. Blocking and hybridization followed standard protocols. The primary antibodies used were monoclonal antibody (mAb) 1H4 (hybridoma cells from A.T.C.C.), anti-HA antibody (a generous gift from Dr S.-C. Cheng, Academia Sinica, Taipei, Taiwan) and anti-tubulin antibody (NeoMarkers), and polyclonal anti-(RNA polymerase II) (N20; Santa Cruz Biotechnology) and anti-(lamin A/C) antibodies (Santa Cruz Biotechnology). Peroxidase-conjugated anti-rabbit or anti-mouse IgGs (Amersham Biosciences) were used as secondary antibodies. Detection was performed using enhanced chemiluminescence (ECL; Amersham Biosciences).

Indirect immunofluorescence

Indirect immunofluorescence was carried out as described previously [31]. The primary antibodies used included polyclonal anti-HA antibody (2 μ g/ml; BabCO) and monoclonal anti-SC35

Figure 1 DRB differentially affects the SR protein profiles in mammalian cells

Cultured mammalian cells were treated or mock-treated with 100 μ M DRB for 3 h. Cell lysate was subjected to Western blot analysis using monoclonal anti-SR-protein antibody 1H4 as a probe. The arrows denote protein induced by DRB treatment. Protein size markers are indicated on the left.

(4.6 μ g/ml; Sigma) and anti-c-Myc (1 μ g/ml, BabCO) antibodies. The secondary antibodies used were the same as described elsewhere [31]. The images were generated with a BioRad MRC1000 confocal microscope.

RESULTS

DRB treatment differentially affects the SR protein profiles in mammalian cells

Since SR proteins function intimately with RNA polymerase II-dependent transcription, it is of interest to understand whether their phosphorylation states could be influenced by the cellular transcription status. We treated cultured mammalian cells with RNA polymerase II inhibitors and subsequently examined SR proteins using mAb 1H4 which recognizes phosphorylated RS domains [32]. Figure 1 shows the SR protein profiles of the cells that were treated or mock-treated with DRB for 3 h. An SR protein of $\approx$ 60 kDa appeared to be induced by DRB in several cell lines, albeit at different levels (Figure 1). This 60 kDa band, however, existed in untreated HEK-293 cells; DRB treatment of HEK-293 cells instead decreased the level of a band immediately below it (Figure 1, lanes 3 and 4). Similar results were obtained in the presence of cycloheximide or using mAb 104, another antibody against phosphorylated RS domains [7], as a probe (results not shown). The former, moreover, suggested that the 60 kDa SR protein was post-translationally modified in response to DRB treatment. However, treatment with another transcription inhibitor actinomycin D had no significant effect on the level or the gel mobility of mAb 1H4-reactive SR proteins (results not shown).

DRB induces hyperphosphorylation of SRp55

Since mAb 1H4 detects prototypical SR proteins [32], we next examined which SR proteins are modified upon DRB treatment. Plasmids expressing HA epitope-tagged SR proteins were constructed and transiently transfected into HeLa cells. Intriguingly, none of the transiently expressed SR proteins changed their mobility on PAGE after DRB treatment (results not shown). We also noted that a substantial amount of overexpressed SR proteins was detected in the soluble fraction when cells were extracted with 0.5 % NP-40, in contrast to

Figure 2 DRB induces a gel mobility shift of SRp55h, but not of any other SR proteins

HeLa cells were transfected with pCEP4-derived vector expressing HA-tagged SR proteins as indicated and cultured in hygromycin-containing medium for 2 weeks. Surviving cells were treated or mock-treated with 100 μ M DRB. Nuclear extracts were subsequently prepared and subjected to Western blot analysis using anti-HA antibody as a probe. The arrow denotes up-shifted SRp55 induced by DRB treatment.

endogenous SR proteins that were resistant to extraction (results not shown). However, these overexpressed SR proteins still remained predominantly localized in the nucleus, as revealed by indirect immunofluorescence (see below for SRp55), indicating that their high solubility was not due to mislocalization in the cytoplasm. We reasoned that a large portion of overexpressed SR proteins were probably not tightly bound to nuclear components such as RNAs or proteins and thus incapable of responding properly to DRB treatment. We therefore selected transfected cells against hygromycin to obtain colonies, in which the ectopically expressed SR proteins showed markedly decreased expression and were resistant to 0.5 % NP-40 extraction (results not shown). Western blotting was then performed using anti-HA antibody, which subsequently showed that, after DRB treatment, only SRp55 shifted its mobility while the other four SR proteins remained unaffected (Figure 2). Moreover, the molecular size of the DRB-induced $\approx$ 60 kDa SR protein (Figure 1) coincided with that of SRp55 (Figure 2, lane 8). Thus SRp55 might be one among prototypical SR proteins whose post-translational modification can be induced by DRB.

Next, we established HeLa cell lines that stably expressed HA-tagged SRp55. Ectopically expressed SRp55 showed reduced gel mobility upon DRB treatment (Figure 3A, lanes 1–4). To determine whether the mobility shift of SRp55 results from hyperphosphorylation, the cell lysates were treated with alkaline phosphatase. After incubation with alkaline phosphatase, SRp55 in untreated cells and its up-shifted form in DRB-treated cells both collapsed to a single faster-migrating form (Figure 3A, lanes 5 and 6). Therefore, SRp55 was readily and heavily phosphorylated in cells but became phosphorylated to a greater extent by DRB treatment.

We further examined whether hyperphosphorylation of SRp55 is specifically induced by transcription inhibition. Transcription inhibitors such as actinomycin D and the isoquinoline sulphonamide derivative H-7 were tested; the latter acts similarly to DRB to promote dephosphorylation of the C-terminal domain of the RNA polymerase II largest subunit [33]. Neither compound showed significant effects on the induction of SRp55 hyperphosphorylation (Figure 3B, lanes 4–7). DRB thus specifically induced hyperphosphorylation of SRp55, which occurred concomitantly with C-terminal domain dephosphorylation (Figure 3B, lanes 1–3). However, the two events could be

Figure 3 DRB specifically induces hyperphosphorylation of ectopically expressed SRp55

(A) Two clones of HeLa cells that stably expressed HA-tagged SRp55 (SRp55.HA) were established. Ectopically expressed SRp55.HA after mock or DRB treatment was detected by anti-HA antibody (lanes 1–4). Nuclear extract prepared from mock- (lane 5) or DRB- (lane 6) treated clone 2 cells was treated with alkaline phosphatase (AP). Hyper-, hypo- and dephosphorylated forms of SRp55 are indicated. (B) SRp55.HA-expressing HeLa cells were mock-treated (lane 1) or treated with DRB (lane 2, 50 μ M; lane 3, 100 μ M), actinomycin D (lane 4, 5 μ g/ml; lane 5, 10 μ g/ml), or H-7 (lane 6, 50 μ M; lane 7, 100 μ M). Nuclear proteins were analysed with anti-HA (upper panel) and anti-RNA polymerase II (lower panel) antibodies. Pol IIo and Pol IIa represent hyper- and hypophosphorylated RNA polymerase II largest subunit, respectively.

uncoupled when cells were treated with H-7 (Figure 3B, lanes 6 and 7).

Hyperphosphorylation of SRp55 occurs at the RS domain and requires RNA-binding activity

While characterizing HA-tagged SRp55-expressing cells, we fortuitously found an N-terminally truncated SRp55 that was still detectable by the antibody against its C-terminal HA tag. The truncated form of SRp55 was highly solubilized with 0.5 % NP-40, as was cytoplasmic protein tubulin (Figure 4A, lane 3). However, immunofluorescence with anti-HA antibody did not detect any signals in the cytoplasm of either transiently or stably expressing cells (Figure 4B), suggesting that HA-tagged SRp55, including the truncated form, was exclusively in the nucleus. We thus suspected that truncated SRp55 bound loosely to nuclear RNA or matrix or interacted poorly with the splicing machinery due to the lack of RRM. In contrast, full-length SRp55 as well as nuclear protein lamins were detected mainly in the residual fraction after extraction with NP-40 (Figure 4A, lane 1). Nevertheless, only full-length but not truncated SRp55 showed retarded migration

upon DRB treatment (Figure 4A, lanes 2 and 4). Thus, truncated SRp55 behaved similarly to transiently overexpressed SRp55, showing no response to DRB signalling effects (see later sections and Figure 9, below).

The above data indicated that RRMs are likely to contribute to the DRB-induced hyperphosphorylation of SRp55; however, the possibility of whether the RNA-binding activity mediates DRB signalling to the RS domain or if phosphorylation instead occurs in the RRMs still remained to be tested. In an attempt to evaluate the importance of RRMs, we constructed vectors for green fluorescent protein- ('GFP-') or c-Myc-tag fusion to the SRp55 RS domain. However, stable clones expressing either fusion protein failed to be established. We therefore went on to explore the role of each modular domain by examining chimaeric SRp55 proteins. The chimaeric protein RRM⁴⁰-RS⁵⁵ showed two bands on PAGE while being expressed ectopically in HeLa cells, probably due to differential phosphorylation (Figure 4C, lane 1). DRB treatment caused a complete shift of the bands to a slower-migrating one (Figure 4C, lane 2), suggesting that a heterologous RNA-binding domain is capable of mediating DRB signalling to the SRp55 RS domain. In contrast, a gel mobility shift was not detected with the chimaera RRM⁵⁵-RS⁴⁰ (Figure 4C, lanes 3 and 4), and is consistent with the above observation that SRp40 failed to become hyperphosphorylated upon DRB treatment (Figure 2). Taken together, our data indicate that hyperphosphorylation of SRp55 induced by DRB occurs at its RS domain and probably requires the RNA-binding activity, but not the specificity.

Hyperphosphorylated SRp55 is located in enlarged speckles

The majority of active transcription sites are localized at the periphery or outside of nuclear speckles [32,34–36]. Phosphorylation of the RS domain allows SR proteins to be recruited from speckles to sites of active transcription [37,38]. While SR proteins are released from speckles following overexpression of SR kinases in cells, an opposite effect has been observed with phosphatase treatment or inhibition of RNA polymerase II transcription [14,17]. Next we continued to examine whether DRB treatment could induce SRp55 relocalization. HA-tagged SRp55 was distributed in the nucleus, with a particularly high concentration in speckles (Figures 5A and 5B). Similar to the observation with endogenous SR proteins [18,19], ectopically expressed SRp55 was relocalized to enlarged speckles upon DRB treatment (Figures 5D and 5E). However, under these conditions, SRp55 was largely hyperphosphorylated (Figures 2–4). Therefore, localization of hyperphosphorylated SRp55 in the speckles contradicted the current belief that SR proteins often disperse in the nucleoplasm while being hyperphosphorylated by SR kinases [18,19].

Overexpression of the Clk/Sty kinase induces degradation of SRp55 through the proteasome pathway

Subsequently we asked whether overproduction of the SR protein kinase SRPK1 or Clk/Sty 1 could cause hyperphosphorylation of the SRp55 RS domain. HeLa cells were then co-transfected with expression vectors for an SR kinase and HA-tagged SRp55, ASF or SC35. Surprisingly, transiently expressed SRp55, but not the other two SR proteins, dramatically declined at the protein level upon co-expression of the Clk/Sty kinase (Figure 6A, lanes 3, 5 and 7). In contrast, overexpression of SRPK1 or a catalytically inactive form (R190K) of Clk/Sty kinase [16] had no effect on the SRp55 protein level (Figure 6A, lanes 2 and 4). The most straightforward explanation for this observation

Figure 4 Hyperphosphorylation of SRp55 occurs at the RS domain and requires RNA-binding activity

(A) SRp55.HA-expressing HeLa cells were mock-treated or treated with 100 μ M DRB for 3 h. Cells were collected and resuspended in a buffer containing 0.5 % NP-40. After centrifugation, the NP-40-solubilized fraction (supernatant; lanes 3 and 4) and the residual fraction (pellet; lanes 1 and 2) were subjected to Western blot analysis using antibodies against the HA epitope (upper panel), lamins and tubulin (lower panel). (B) Cellular distribution of transiently or stably expressed SRp55.HA was detected by indirect immunofluorescence with anti-HA antibody. Cells visualized by phase-contrast microscopy are also shown. (C) HeLa cells were transfected with vectors expressing HA-tagged chimeric protein RRM⁴⁰-RS⁵⁵ (lanes 1 and 2) or RRM⁵⁵-RS⁴⁰ (lanes 3 and 4). Hygromycin-resistant cells were mock-treated or treated with DRB. HA-tagged SR proteins were detected with anti-HA antibody.

Figure 5 Hyperphosphorylated SRp55 distributes in enlarged nuclear speckles

SRp55.HA-expressing HeLa cells were mock-treated (A–C) or treated with DRB (D–F). Indirect immunofluorescence was performed to detect the distribution of ectopically expressed SRp55.HA (A and D) and anti-SC35-antibody-reactive SR proteins (B and E). Cells visualized by phase contrast microscopy are shown (C and F).

is that the Clk/Sty kinase may phosphorylate SRp55 and thus subsequently cause its destabilization (see below), although other possibilities remain as well. Therefore, to determine whether

SRp55 degradation is dependent on the ubiquitin-proteasome pathway, cells were co-transfected with the vectors expressing HA-tagged SRp55 and Clk/Sty for 8 h, and then treated for 15 h

Figure 6 Overexpression of Clk/Sty induces degradation of SRp55 through the proteasome pathway

(A) HeLa cells were co-transfected with an empty vector (lane 1) or expression vector for an SR kinase [SRPK1, lane 2; wild-type Clk/Sty, lane 3; mutant Clk/Sty K190R (KR), lane 4] and vector expressing HA-tagged SRp55 (lanes 1–4), ASF (lanes 5 and 6) or SC35 (lanes 7 and 8). HA-tagged SR proteins were detected with anti-HA antibody. (B) HeLa cells were co-transfected with expression vectors for HA-tagged SRp55 and wild-type (lanes 1 and 2) or mutant (lanes 3 and 4) Clk/Sty. At 8 h post-transfection, 10 μ M lactacystin was added to the culture medium for another 15 h. Nuclear extract was subjected to Western blot analysis with anti-HA (upper panel) and anti-lamin (lower panel) antibodies.

with lactacystin, a specific proteasome inhibitor. Figure 6(B) shows that Clk/Sty-mediated decline of SRp55 was markedly blocked by lactacystin (Figure 6B, lane 2), suggesting that SRp55 is targeted for proteolysis by the proteasome. We therefore hypothesize that the Clk/Sty kinase phosphorylates SRp55 at the RS domain and subsequently triggers proteasome-mediated degradation of SRp55.

To exclude the possibility that SRp55 becomes susceptible to Clk/Sty-mediated degradation only when it is overproduced, HeLa cells that stably express HA-tagged SRp55 were transfected with the vectors for the Clk/Sty kinase. Indirect immunofluorescence was performed to detect ectopically expressed SRp55 in kinase-overproducing cells. As shown in Figure 7, HA-tagged SRp55 was undetectable in the cells expressing wild-type Clk/Sty kinase (Figures 7A and 7B). In contrast, signals reactive to anti-SC35 antibody still remained detectable under these conditions (Figures 7G and 7H). Also consistent with the above result was that no reduction of SRp55 was observed in cells that

overproduced mutant Clk/Sty (Figures 7D and 7E). Therefore, ectopically expressed SRp55 can be specifically destabilized in the presence of excess Clk/Sty kinase.

The entire SRp55 RS domain is required for Clk/Sty-mediated degradation

Next we went on to examine whether a degradation signal is located within the SRp55 RS domain. The entire SRp55 RS domain comprises three parts: the N-terminal long RS region (RS1), the middle proline-rich region and the C-terminal short RS region (RS2; Figure 8A). Initially, two truncated SRp55 proteins were individually co-expressed with wild-type or kinase-inactive Clk/Sty in HeLa cells, and their expression level was measured by Western blotting. Deletion of RS2 substantially increased the level of truncated SRp55 in the presence of wild-type Clk/Sty (Δ C21; Figure 8B, lanes 3 and 4). A further removal of the proline-rich sequence almost completely abolished the Clk/Sty-mediated destabilization of SRp55 (Δ C43; Figure 8B, lanes 5 and 6). Therefore a degradation signal most likely resides in the C-terminal 43-amino acid region (C43) of SRp55.

To further test whether the C43 sequence can destabilize a heterologous protein, we placed it at the C-terminus of ASF (Figure 8A). However, overexpression of wild-type Clk/Sty did not destabilize the ASF-C43 fusion (Figure 8B, lane 7). Therefore the C43 domain appeared not to be sufficient for Clk/Sty-mediated protein degradation. We also noted that the SRp55 RS1 domain remarkably contains many lysine/serine dipeptides, of which the serine residues can be phosphorylated by Clk/Sty but not by SRPK1 [22]. Thus it is possible that overexpressed Clk/Sty kinase extensively phosphorylates the RS1 domain of SRp55, and thereby triggers degradation that is initiated by the C43 region. If this is true, the whole SRp55 RS domain might be critical for Clk/Sty-mediated degradation. Therefore, the RS domain of ASF was entirely replaced by that of SRp55 and the resulting chimera RRM^{ASF}-RS⁵⁵ was transiently co-expressed with Clk/Sty. The level of the chimaeric protein was markedly down-regulated by the presence of excess wild-type Clk/Sty (Figure 8B, lane 9). This result thus supports our hypothesis that the C43 region bears a destabilization signal that is regulated by Clk/Sty probably through phosphorylation of the lysine/serine-rich RS1 domain. Taken together, the entire RS domain is essential for Clk/Sty-mediated SRp55 degradation, but its N-terminal RS1 and C-terminal C43 subdomains play distinct roles.

DISCUSSION

Numerous lines of evidence have shown that pre-mRNA splicing can be regulated via modulation of the level, phosphorylation state or localization of SR proteins [11–14,20,23,26–28]. However, since all prototypical SR proteins share the same domain structure [2], the question as to how they are individually targeted for regulation has remained unanswered. In this study, we demonstrate that DRB specifically induces hyperphosphorylation of SRp55 and its relocalization to nuclear speckles. More interestingly, overexpression of SR kinase Clk/Sty specifically results in proteolysis of SRp55 by the proteasome. Thus this study reveals novel effects of hyperphosphorylation on the fate of SRp55 in cells.

DRB is a potent inhibitor of kinases that phosphorylate the C-terminal domain of RNA polymerase II and thus inhibits transcription elongation [33]. Moreover DRB can inhibit casein kinase II that has numerous cellular substrates [39]. Hyperphosphorylation of SRp55 in response to DRB treatment may result

Figure 7 Ectopically expressed SRp55 is degraded upon overexpression of Clk/Sty

SRp55.HA-expressing HeLa cells were transfected with expression vector for Myc-tagged wild-type Clk/Sty (**A–C** and **G–I**) or its K190R mutant (**D–F**). Distribution of HA-tagged SRp55 (**B** and **E**), wild-type (**A** and **G**) and mutant (**D**) Clk/Sty, and anti-SC35-antibody-reactive SR protein (**H**) was detected using indirect immunofluorescence. The images produced by phase contrast microscopy are shown (**C**, **F** and **I**).

either from activation of an SR kinase or inactivation of a phosphatase. Such enzyme activity was not induced by transcription inhibitors actinomycin D or H-7 (Figure 3) and appeared to vary substantially between the cell lines examined (Figure 1). No significant change at the phosphorylation level of SRp55 was detected at different stages of the cell cycle (results not shown). The activity that directly modulates the phosphorylation level of SRp55 in response to DRB still remains to be identified. Moreover, neither N-terminally truncated (Figure 4) nor overexpressed (results not shown) SRp55 became hyperphosphorylated after DRB treatment. However, DRB-induced hyperphosphorylation can occur with the SRp55 RS domain fused to a heterologous RNA-binding domain (Figure 4). Thus, the RNA-binding domain as well as a limited expression level appears to be important for mediating DRB signalling to the SRp55 RS domain. DRB treatment indeed caused relocalization of ectopically expressed SRp55 to the nuclear speckles (Figure 5), indicating that it is withdrawn from the transcription sites to its reservoirs. We therefore hypothesize that, while functioning in co-transcriptional RNA processing, SRp55 is capable of responding to signals from DRB treatment (Figure 9).

Continuous movement of splicing factors from speckles to active transcription sites ceases upon inhibition of RNA polymerase II transcription, and thereby nuclear speckles become prominent concomitant with the decrease of the nucleoplasmic signals of splicing factors [18,19,32,37]. Consistently, ectopically

expressed SRp55 was mainly relocalized to enlarged speckles when cells were treated with DRB (Figure 5). However, under this condition, the majority of SRp55 became hyperphosphorylated (Figures 2–4). At present, the possibility that hyperphosphorylated SRp55 is selectively repelled from enlarged speckles cannot be completely excluded. If hyperphosphorylated SRp55 indeed relocates to speckles, the result is then in conflict with the currently held belief that phosphorylation of SR proteins often renders their targeting to sites of transcription and diffusion in the nucleoplasm [38]. Although it is still possible that targeting of SRp55 to transcription sites involves a certain level of RS domain phosphorylation, hyperphosphorylation of SRp55 induced by DRB could, in contrast, allow its tight association with components in speckles. On the other hand, speckle enlargement induced by DRB may involve an as-yet-unidentified mechanism that may not be simply via modulation of the phosphorylation level of SR proteins. Nevertheless, the questions as to how trafficking of splicing factors within the nucleus co-ordinates with transcription and how such trafficking is modulated by the phosphorylation status of splicing factors require further detailed analysis.

A notable finding here is that overexpression of the Clk/Sty kinase specifically induced the degradation of SRp55, but not ASF or SC35, probably via the ubiquitin-mediated proteolytic pathway (Figures 6 and 7). The very C-terminal region, C43, of SRp55 may serve as a conditional destabilization signal that is

Figure 8 The roles of the SRp55 RS subdomain in Clk/Sty-mediated degradation

(A) Domain structures of full-length (FL) and truncated (Δ) SRp55 and chimaeric proteins. (B) HeLa cells were co-transfected with expression vectors for HA-tagged SRp55 or its derivatives as indicated and wild-type (lanes 1, 3, 5, 7 and 9) or mutant (KR; lanes 2, 4, 6, 8 and 10) Clk/Sty kinase. SRp55 and its derivative proteins were detected with anti-HA antibody and their level was measured by a densitometer. Ratios (KR/Clk) represent the relative level of each protein in mutant Clk/Sty-coexpressing cells over that in cells expressing wild-type Clk/Sty; means were obtained from 2 or 3 independent experiments.

Figure 9 Model for differential effects of hyperphosphorylation on SRp55

Splicing factor SRp55 functions in co-transcriptional pre-mRNA splicing. When transcription is inhibited by DRB, SRp55 becomes hyperphosphorylated at the RS domain and relocates to enlarged nuclear speckles. Since DRB treatment had no effect on truncated (RRM-lacking) or overexpressed SRp55, it is possible that only functionally active SRp55 is responsible for DRB-induced hyperphosphorylation. On the other hand, overexpression of the Clk/Sty kinase results in destabilization of SRp55 by the ubiquitin-proteasome pathway. CTD, C-terminal domain of the RNA polymerase II (pol II) largest subunit.

likely to be regulated by the adjacent RS domain (Figure 8). The reasons that make this conclusion compelling are as follows. First, deletion of the C43 domain greatly stabilized SRp55 in the presence of excess Clk/Sty kinase. Second, C43 was insufficient by itself to activate protein degradation but appeared to act synergistically with the SRp55 RS1 domain to trigger degradation. Third, excess Clk/Sty kinase activated

SRp55 degradation, possibly by prior phosphorylation of the RS1 domain, since it bears several copies of lysine/serine dipeptides that could serve as a substrate for Clk/Sty but not SRPK1 [22]. However, alternative explanations for our observations cannot be excluded. For example, C43 and RS1 reverse their roles in the degradation of SRp55, or the Clk/Sty kinase acts indirectly to modulate the stability of SRp55. Moreover, the Clk/Sty-mediated pathway was able to target ectopically expressed SRp55 in both transiently and stably expressing cells (Figures 6 and 7). This differs from the scenario of DRB-induced hyperphosphorylation that occurs only on endogenous and stably expressed SRp55 (Figures 1 and 2). More interestingly, the SRp55 destabilization signal appears to be regulated by its adjacent RS domain, probably via phosphorylation (Figure 8). The possibilities as to whether the destabilization signal is unmasked or the ubiquitin ligase is actively recruited [40] following the Clk/Sty-mediated phosphorylation still remain to be characterized. Nevertheless, our understanding of Clk/Sty-mediated SRp55 degradation remains incipient and its molecular mechanism requires further investigation.

It has been previously reported that, during apoptotic cell death, several RNA processing proteins including U1-specific 70 kDa protein and heterogeneous ribonucleoprotein C ('hnRNP C') are cleaved by caspases [41]. The present study shows, for the first time, that SRp55 is specifically degraded by the proteasome, most likely via the ubiquitin-dependent pathway. The ubiquitin system targets many proteins including those involved in transcription, cellular signalling and the cell cycle, and thus plays a central role in the control of many basic cellular processes [40]. However, the functional significance of SRp55 degradation that occurs when the level of the Clk/Sty kinase is elevated remains to be resolved. It will be of particular interest to explore whether alternative splicing is affected when SRp55 is down-regulated and whether any external stimuli can induce destruction of SRp55 via activation of Clk/Sty. Finally, it is notable that *Drosophila* contains fewer SR protein family members but that its SRp55 homologue, B52, is known to play a critical role in development [42]. Recently, an expression profile revealed that transcripts encoding SRp55 and SRp20 are enriched in embryonic and adult neural and haematopoietic stem cells [43], suggesting that post-transcriptional regulation mediated by these SR proteins such as alternative splicing, is critical for stem cells in self-renewal or differentiation.

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