Increased Serine-Arginine (SR) Protein Phosphorylation Changes Pre-mRNA Splicing in Hypoxia*

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The removal of introns from mRNA precursors (pre-mRNAs) is an essential step in eukaryotic gene expression. The splicing machinery heavily contributes to biological complexity and especially to the ability of cells to adapt to altered cellular conditions. Inhibitory PAS domain protein (IPAS), a dominant negative regulator of hypoxia-inducible gene expression, is generated from hypoxia inducible transcription factor-3α (HIF-3α) pre-mRNA by an alternative splicing mechanism. Inactivation of the IPAS transcript in mice leads to the neo-vascularization of the cornea, suggesting that IPAS is an important regulator of anti-angiogenesis in this tissue. For the first time we demonstrate that serine-arginine (SR) proteins are involved in oxygen tension-dependent changes in pre-mRNA splicing. SR proteins isolated from hypoxic cells differentially interact with RNA (compared with proteins isolated from cells cultured under normoxic conditions). They possess the differential ability to activate hypoxia-dependent splice sites, and they are more phosphorylated than those isolated from normoxic HeLa cells. We also show that expression of SR protein kinases (CLK1, SRPK1, SRPK2) in hypoxic cells is elevated at mRNA and protein levels. Increased expression of CLK1 kinase is regulated by HIFs. Reduction of CLK1 cellular expression levels reduces hypoxia-dependent full-length carbonic anhydrase IX (CAIX) mRNA and CAIX protein formation and changes hypoxia-dependent cysteine-rich angiogenic inducer 61 (Cyr61) mRNA isoform formation profiles.

Background: Splicing machinery heavily contributes to the ability of cells to adapt to hypoxic conditions.

Results: SR proteins become hyperphosphorylated in hypoxia by HIF-1-dependent increase in SR protein kinase expression.

Conclusion: SR proteins are one of hypoxia-dependent pre-mRNA splicing regulators.

Significance: This is the first elucidation of factors involved in hypoxia-dependent splicing regulation.
Pre-mRNA splicing in 16 different genes is changed in human umbilical vein endothelial cells, cultivated under hypoxia mimicking condition (8). The mechanism underlying oxygen tension-dependent changes in splicing remains unknown.

RNA splicing takes place in the nucleus and occurs either co- or post-translationally. Noncoding sequences (introns) in nuclear mRNA precursors (pre-mRNA) are removed by dedicated splicing machinery. Coding sequences (exons) are joined to generate the mature mRNA that is exported to the cytoplasm and translated into protein. Splicing events are tissuem-specific (5, 9). This process plays an important role in cellular differentiation and development. It is now clear that the splicing machinery heavily contributes to biological complexity especially to the ability of cells to adapt to different developmental stages and altered cellular conditions (9, 10). Recent studies indicate that the components of the spliceosome themselves are able to regulate pre-mRNA splicing (5, 9, 11).

Serine-arginine (SR) proteins are a highly conserved family of splicing factors that are present throughout metazoans. They have diverse roles in constitutive and regulated splicing. SR proteins are essential splicing factors in vitro, as they are required for an early step(s) in the process of spliceosome assembly (12). All SR proteins have a similar bipartite structure composed of two functional domains: an N-terminal RNA binding domain, comprised of multiple RNA-recognition motifs and a C-terminal arginine-serine-rich (RS) domain. By interacting with the U1 70K protein, a component of the U1 small nuclear ribonucleoprotein particle (snRNP), SR proteins interacting with the U1 70K protein, a component of the U1 motif and a C-terminal arginine-serine-rich (RS) domain. By binding to the polypyrimidine tract of the intron (12, 14, 15). SR proteins are involved in stabilization of U2AF subunit of splicing factor U2AF) at the splice sites (14). SR proteins also participate in splicing control. In the case of alternative splice sites, they likely function by recruiting U1 small nuclear ribonucleoprotein to alternative splice sites. There is evidence that SR proteins are involved in stabilization of U2AF binding to the polypyrimidine tract of the intron (12, 14, 15).

A number of studies have shown that all activities of SR proteins are modulated by phosphorylation within the RS domain. It has been reported that RS domain phosphorylation influences the subcellular localization of SR proteins as well as protein–protein interactions (16, 17). Hypo- and hyper-phosphorylated SR proteins are inactive in splicing assays (18, 19). Modulation of phosphorylation of SR proteins is used to control pre-mRNA splicing in adenovirus infection and in early development in the nematode Ascaris lumbricoides (19, 20). It has also been shown that specific SR protein phosphorylation levels are modulated during the response to heat shock (21).

Multiple protein kinases are involved in SR protein phosphorylation. Among these SR protein kinases the best-characterized ones are members of SR protein kinase (SRPK) and CLK/STY families. Mammalian cells express two SRPKs and four members of the CLK/STY family of kinases. In contrast to SRPK kinases, which phosphorylate only a limited range of phosphorylation sites on SR proteins, the CLK kinases are able to phosphorylate the entire RS domain producing a hyperphosphorylated form of SR proteins (17, 22–24).

A splice variant of mouse HIF-3, which is an inhibitory PAS domain protein (IPAS), was initially detected in mouse cornea epithelial cells. IPAS inhibits HIF-1 dimerization with ARNT (aryl hydrocarbon (dioxin) receptor (AhR) nuclear translocator protein), thus inhibiting HIF-1-dependent transcriptional activation. IPAS mRNA is generated by alternative splicing of the HIF-3 locus, and its production is strictly regulated by hypoxia. In addition to the unique exons 1a and 16, the IPAS mRNA species contains a third unique exon 4a. Moreover, an acceptor site competition mechanism generates not only a 14-nucleotide 5′ deletion of exon 3 but also an 87-nucleotide 3′ deletion of exon 6. The utilization of exon 4a together with the 5′ deletion of exon 3 results in a reading frameshift, which is a unique feature of the IPAS mRNA (25, 26).

In the current study we demonstrate that cells, in response to hypoxia, change alternative pre-mRNA splicing. This is done by changing SR protein activity via HIF-1-dependent SR protein kinase expression enchancement thus changing SR protein phosphorylation levels. Functionally, SR proteins are required for basic, constitutive pre-mRNA splicing as well as for numerous alternative splicing events (12). In conclusion, one of mechanisms by which hypoxia alters pre-mRNA splicing pattern is by altering SR protein activity.

**Materials and Methods**

**Cells, Nuclear Extracts, Antibodies, and Pre-mRNA Substrates**—HeLa cells were cultured in DMEM media under either normoxic or hypoxic (24 h at 1% O2, 5% CO2, and 94% N2, in an Invitro200 hypoxic work station (Ruskin Technologies)) conditions, and nuclear extracts were prepared (27). For Western blots anti-SR (LifeSpan Biosciences), mAb104, anti-CLK1 (Abcam), anti-SRPK1 (Abcam), anti-SRPK2 (Abcam), anti-HIF-1α (GeneTex) antibodies were used. Constructs for in vitro pre-mRNA splicing were constructed from the mouse HIF-3α gene. The HIF-3α construct spanned exon 3, part of intron 3, and part of intron 4 and exon 4. The IPAS construct spanned exon 3, a shortened intron 3, and exon 4a. The HIF-3α constructs were obtained by PCR using appropriate primer pairs: P1/P2 (5′-d(AAGGATCTAGAAGAGCCACTGGACGCCTGCG)-3′/5′-d(TTCCCAAGCGTCTCAGGAGTGTCG)-3′)’ and P3/P4 (5′-d(AAGGAAAGCTTGGAGAGCAGACATATGACTCTCG)-3′/5′-d(TTCCCTCTCGAGTCTTTGAGATGGCGGCTTG)-3′). The IPAS construct was obtained using P1/P2 and P5/P6 (5′-d(AAGGAAAGCTTGGAGAGCAGACATATGACTCTCG)-3′/5′-d(TTCCCTCTCGAGTCTTTGAGATGGCGGCTTG)-3′). Primer pairs. PCR fragments (HIF-3α and IPAS) were cloned into plasmid vector II KS (+) plasmid DNA (ThermoFisher Scientific) through XbaI/HindIII and HindIII/XhoI restriction endonuclease sites, respectively. The β-globin splicing pre-mRNA construct was obtained by PCR from plasmid DNA containing rabbit β-globin gene using a P7/P8 (5′-d(AATTACGACTCTAGTATAGAATAACGACTTCGGGAGTCTGCG)-3′/5′-d(GAGGACAGTGCCCATCAGGAAAATTGGTACCAAGGGAGTCTGCG)-3′) primer pair. PCR fragments (HIF-3α and IPAS) were cloned into plasmid vector II KS (+) plasmid DNA (ThermoFisher Scientific) through XbaI/HindIII and HindIII/XhoI restriction endonuclease sites, respectively. The β-globin splicing pre-mRNA construct was obtained by PCR from plasmid DNA containing rabbit β-globin gene using a P7/P8 (5′-d(AATTACGACTCTAGTATAGAATAACGACTTCGGGAGTCTGCG)-3′/5′-d(GAGGACAGTGCCCATCAGGAAAATTGGTACCAAGGGAGTCTGCG)-3′) primer pair. **Materials and Methods**

**T7 Transcription and Pre-mRNA Splicing in Vitro**—Labeled pre-mRNAs from the plasmids were generated in an in vitro transcription reaction using T7 RNA polymerase (ThermoFisher Scientific) and [3P]CTP (PerkinElmer Life Sciences).
Transcription reaction products were purified. Splicing reactions (25 μl) contained ~10 fmol in vitro transcribed, capped, and 33P-labeled pre-mRNA, 20% HeLa nuclear extract, 2.6% (w/v) polyvinyl alcohol, 2.8 mM MgCl2, 2 mM ATP, 20 mM phosphocreatine, and buffer D (20% (w/v) glycerol, 20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.3 mM PMSF). Reactions were incubated 0.5–2 h at 30 °C followed by proteinase K digestion (40 μg) (ThermoFisher Scientific) for 30 min at 37 °C, extracted with phenol-chloroform, and ethanol-precipitated. Pre-mRNA splicing reaction products were resolved on 8% denaturing polyacrylamide gels.

**UV Cross-linking, “Fishing Out” Bound Proteins, and Western Blotting**—UV cross-linking was performed as earlier described (28). Nuclear extracts (100 μg proteins) were incubated with 25–40 fmol of 33P and biotin double-labeled RNA in splicing buffer lacking creatinine phosphate (25 μl total volume). The reaction mixture was exposed to 254 nm for 15 min on ice. Bound pre-mRNA proteins were separated from unbound proteins using streptavidin beads (Invitrogen), digested with 10 μg of RNase A (ThermoFisher Scientific) at 37 °C for 30 min, and separated on a 10% SDS-polyacrylamide gel. If required, the separated proteins were transferred to a nitrocellulose membrane. The membranes were incubated with appropriate (anti-SR (LifeSpan Biosciences) or mAb104, anti-CAIX (Biosciences)) antibodies, washed, and incubated with secondary antibody conjugated with HRP (Dako). The membranes developed using ECL reagent (GE Healthcare).

**SR Protein Isolation from HeLa Cells**—SR proteins from HeLa cells cultured under hypoxic (1% oxygen) or normoxic (21% oxygen) conditions were isolated as described earlier (29).

**Isolation of Kinase mRNA, cDNA Synthesis, and RT-PCR**—Kinase mRNA was isolated from HeLa cells using a Quick RNA Miniprep kit (Zymo Research). Using the RevertAid H Minus First Stand cDNA Synthesis kit (ThermoFisher Scientific) and specific primers, mRNA was converted to cDNA. RT-PCR was performed using specific primers for CLK1 (5′-d(ATTTTTGTTGGGTGCGCGA)-3′/5′-d(TCCTTCGGTACCTCCTCCA)-3′), for SRPK1 (5′-d(GGACAAAGCCCAAAGGAAA-GATCCTTCGGTACCTCCTCCA)-3′), for SRPK2 (5′-d(GGACAAAGCCCAAAGGAAATTCCTTCGGTACCTCCTCCA)-3′), and for 18S RNA analysis (5′-d(AACTCACTGAAGATGAGGTG)-3′/5′-d(CAGACAGGTTTTGGGATGCTTACC)-3′). The RT-PCR products were analyzed on a 1.5% agarose gel in Tris borate-EDTA buffer.

**Two-dimensional Gel Analysis**—50 μg of purified SR proteins prepared from normoxic or hypoxic HeLa cells were subjected to two-dimensional analysis with IPRUNNER strip, pH 3–10, using a ZOOM IPRUNNER Mini-Cell (Invitrogen) apparatus according to manufacturer’s instructions. Isoelectric focusing (200 V for 20 min, 350 V for 10 min, 500 V for 4 h, 2000 V for 2 h) was performed at 20 °C after a 12-h rehydration step. After the second-dimension separation on 12% polyacrylamide gel, proteins were transferred onto a nitrocellulose membrane, and blots were probed with anti-SR antibody (LifeSpan Biosciences).

**Chromatin Immunoprecipitation (ChIP Assay)**—Cells were fixed with 1% formaldehyde at room temperature for 10 min and collected in ice-cold PBS, washed with Buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 6.5) and Buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 6.5) and lysed using lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0, 1 × protease inhibitor mixture (Roche Applied Science)). The obtained nuclear extract was sonicated in Bioruptor UCD-200TM (Diagenode). Samples were cleared by centrifugation. The supernatant was diluted with Dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 × protease inhibitor mixture (Roche Applied Science)) 1:10. Immunoclearing was performed by incubating soluble chromatin with sheared herring sperm DNA, rabbit anti-human IgG, and protein-G-Sepharose 50% slurry. The supernatant was incubated with HIF-1α-specific antibodies (GeneTex) overnight at +4 °C. The antibody-bound DNA was harvested using protein-G-Sepharose 50% slurry, washed with TSE I buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), TSE II Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), Buffer III (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and Tris-EDTA buffer and eluted three times with Elution buffer (1% SDS, 0.1 M NaHCO3). The eluates were pooled and incubated at 65 °C overnight to reverse the cross-link. DNA fragments were purified using the GeneJet PCR purification kit (ThermoFisher Scientific) according to the manufacturer’s instructions. Quantitative PCR was done with primer pair (CLK1-HRE forward 5′-d(CGTACAGGGTCTGACGAACTCTC)-3′/CLK1-HRE reverse d(5′-GCCTCACCTCTCTCCTTCTGC-3′)) using Maxima SYBR Green qPCR Master mix (ThermoFisher Scientific) on Qiagene Rotor-Gene 6000.

**HeLa Cell Transfections with CLK1 siRNA or Treatment with CLK Kinase Inhibitor**—siRNA (Life Technologies) against CLK1 was used for inhibition of endogens CLK1 mRNA in HeLa cells. An additional siRNA as the negative control was obtained from Qiagen. siRNAs were reconstituted under RNase-free conditions according to the manufacturer’s protocol. HeLa cells were transfected with the sense and control siRNAs at 60% confluence with RNAiMAX Transfection Reagent (Life Technologies) in the appropriate culture medium without antibiotics containing 10% FBS according to the supplier’s protocol. After transfection, cells were allowed to recover for 18 h and subsequently exposed for 24 h to normoxia (21% O2) or hypoxia (1% O2).

HeLa Cells Suspended in 2 ml of DMEM Medium Were Plated on 6-Well Dishes. TG003 was dissolved in DMSO and added to cells till 10 μM final concentration. Cells were exposed for 24 h to normoxia (21% O2) or hypoxia (1% O2).

After siRNA experiments HeLa cells were collected. Using a Quick RNA Miniprep kit (Zymo Research), total RNA was isolated. The RNA samples were then converted to cDNA with the RevertAid H Minus First Stand cDNA Synthesis kit (ThermoFisher Scientific). RT-PCR was performed using primers specific to endogenous CLK1 (CLK1.1 forward/CLK1 reverse 5′-d(ATTTTTGTTGGGTGCGCGA)-3′/5′-d(TCCTTCGGTACCTCCTC)-3′), full-length (FL) carbonic anhydrase IX (CAIX) (CA9-ex7 forward/CA9-ex8 reverse 5′-d(TAATCTGCAACTCTGACCACTC)-3′), and alternatively spliced (AS) CAIX (CA9-ex7
forward/CA9-ex7/10 reverse 5′-d(TATCTGCACCTCTGCCCCTCTG)-3′/5′-d(CTAGGATGTCACCTGCTTAGCACTC)-3′ transcripts. Primers used for cysteine-rich angiogenic inducer 61 (Cyr61) pre-mRNA splicing analysis were described in Hirschfeld et al. (30). The RT-PCR products were analyzed on a 1% agarose gel.

Results

We used mouse HIF-3α pre-mRNA as a model system for our studies. This pre-mRNA is spliced in a hypoxia-dependent manner producing HIF-3α (in normoxia and hypoxia) and IPAS (mainly in hypoxia) pre-mRNAs (Fig. 1A). We generated constructs spanning the common exon 3 5′ splice site and either the unique splice sites of exon 4 (HIF-3α 3′-specific splice site) or exon 4a (IPAS 3′-specific splice site). Sometimes, in normoxic nuclear extracts in which we did not detect IPAS splicing product, we thought the addition of splicing enhancer would give us a stable, detectable IPAS splicing product. The constructs were generated with or without a splicing-enhancer sequence (31) in the 3′ end of second exon (HIF-3α-U1 and IPAS-U1) (Fig. 1, 1B, lanes 3 and 4). This experiment demonstrates that just the addition of a splicing enhancer sequence to the second exon converts IPAS pre-mRNA from very poorly spliced RNA to a quite efficiently spliced construct. IPAS and HIF3α constructs with splicing enhancers are spliced too efficiently, and it

FIGURE 1. IPAS and HIF-3α constructs are spliced differently in nuclear extracts prepared from HeLa cells cultured under normoxic conditions. A, schematic representation of HIF-3α pre-mRNA. B, schematic representation of constructs used in in vitro splicing assays. Branch point is indicated by an asterisk (*). C, IPAS and HIF-3α pre-mRNAs splicing in in vitro splicing reactions in nuclear extracts prepared from normoxic cells. D, average of HIF-3α and IPAS splicing efficiencies (from three experiments) quantified by phosphorimaging.
would be difficult to interpret results. In further hypoxia-dependent pre-mRNA splicing studies we have used HIF-3α and IPAS constructs without splicing enhancer sequences.

Next we tested splicing of the HIF-3α and IPAS constructs in nuclear extracts from hypoxic cells. The splicing product of the HIF-3α construct in these extracts was barely detectable (Fig. 2, A and D, lanes 2–4). The opposite result was obtained when the IPAS construct was tested in the in vitro splicing assay in the same extracts (Fig. 2, B and E, lanes 2–4). When weakly spliced in hypoxic extracts, IPAS product formation was already observed after 1 h of reaction (Fig. 2, B and E, lanes 3). Bands corresponding to the lariat exon 4a intermediate and exon 3 (Fig. 2, B and E, lanes 3) formation can be seen, indicating that, in contrast to splicing in normoxic extracts, hypoxic nuclear extracts are capable of activating weak 3’ splice site usage. In control experiments β-globin pre-mRNA was spliced with 20–35% efficiency in nuclear extracts prepared under normoxic conditions (Fig. 2, C and F, lane 1). In hypoxic nuclear extracts splicing efficiency of β-globin pre-mRNA decreased ∼2-fold compared with normoxic extracts (Fig. 2, C and F, lane 2). These results indicate that nuclear extracts (prepared from HeLa cells cultured under hypoxic conditions) possess a weaker capability to activate a strong β-globin 3’ splice site (as opposed to normoxic nuclear extracts) and activate weak splice site usage within the HIF3α construct.

SR Proteins Isolated from Hypoxic Cells Interact with RNA and Activate Splicing Differently—As nuclear extracts from normoxic or hypoxic cells activate pre-mRNA splicing differently, we examined the interaction of HIF-3α pre-mRNA with proteins from these extracts in UV cross-linking experiments. We observed that a protein from hypoxic nuclear extracts interacts with RNA more efficiently compared with proteins from normoxic extracts (Fig. 2 G). To define differentially interacting proteins, HIF-3α pre-mRNA was double-labeled (32P and biotin) HIF-3α pre-mRNA. Fishing controls from NEnor (lane 1) and NEhypox (lane 2) with just 32P-labeled RNA are shown.
Western blot analysis of incubation with nuclear extracts from normoxic or hypoxic cells and affinity precipitation using streptavidin beads revealed that SR proteins in nuclear extracts from hypoxic cells interacted more strongly with RNA as compared with those from normoxic nuclear extract (Fig. 2H, lanes 3 and 4).

We isolated (29) SR proteins from HeLa cells cultured under normoxic or hypoxic conditions, respectively (SRnor or normoxic SR and SRhpx or hypoxic SR, respectively) and tested their ability to activate HIF-3α/H9251, IPAS, and β-globin pre-mRNA splicing. The addition of SRnor to the splicing reaction in normoxic nuclear extract activated β-globin and HIF-3α/H9251 construct splicing (Fig. 3, A and D, lanes 1–3 and 8–11, respectively). The addition of SRnor to normoxic nuclear extracts did not activate IPAS construct splicing (Fig. 3, A and D, lanes 4–7).

The addition of SRhpx to NEnor activated splicing of all three tested constructs (HIF-3α, IPAS, and β-globin) (Fig. 3, B and E, C and F), demonstrating that SRhpx possesses a stronger ability to activate splicing as compared with SRnor. Normoxic SR proteins (Fig. 4, A and D, lanes 2) activated HIF-3α pre-mRNA splicing (Fig. 4, A and D, lanes 3 and 4) and slightly activated IPAS transcript splicing (Fig. 4, B and E, lanes 1–3). Finally, the addition of SRnor (Fig. 4, C and F, lanes 3–5) or SRhpx (Fig. 4, C and F, lanes 6–8) proteins to hypoxic nuclear extracts had almost no effect on β-globin pre-mRNA splicing efficiency. These results indicate that SR proteins from hypoxic cells, compared with SR proteins from normoxic cells, possess different capacities to activate splicing of certain splice sites.

Preincubated Normoxic SR Proteins in Hypoxic Nuclear Extract Lose Their Ability to Activate HIF-3α Pre-mRNA Splicing—As hypoxic cellular effects are reversible, it may be possible that the distinct SR protein activities in normoxic and hypoxic cells might be reversible. To test this hypothesis SRnor proteins were either added immediately into the splicing reac-

**FIGURE 3. Influence of SRnor and SRhpx on HIF-3α, IPAS, and β-globin pre-mRNAs splicing in normoxic HeLa cell nuclear extracts.** A, splicing of β-globin, IPAS, and HIF-3α transcripts in NEnor (lanes 1, 4, and 8, respectively) or with increasing amounts (0.25–0.75 μg) of SRnor (lanes 2 and 3 (β-globin), lanes 5–7 (IPAS), and lanes 9–11 (HIF-3α)), B, splicing of IPAS and HIF-3α transcripts in NEnor (lanes 1 and 5, respectively) or with increasing amounts (0.25–0.75 μg) of SRnor (lanes 2–4 and 6–8, respectively). C, splicing of β-globin transcript in NEnor (lane 1) or with increasing amounts (0.25–0.5 μg) of SRhpx (lanes 2 and 3, respectively). D, average β-globin (lanes 1–3), IPAS (lanes 4–5), and HIF-3α (lanes 8–10) construct splicing efficiencies with SRnor (from three experiments) quantified by phosphorimaging. E, average IPAS (lanes 1–4) and HIF-3α (lanes 5–7) construct splicing efficiencies with SRhpx (from three experiments) quantified by phosphorimaging. F, average β-globin splicing efficiencies with SRhpx (from three experiments) quantified by phosphorimaging.
tion (Fig. 5, A and B, lanes 1–4) or, before addition of HIF-3α pre-mRNA, were pre-incubated in normoxic or hypoxic nuclear extracts for 20 min at 30 °C temperature (Fig. 5, A and B, lanes 5–8). The splicing results revealed that addition of SRnor into the splicing reaction in NEnor (lane 1) or with increasing amounts (0.25–0.5 μg) of SRnor (lanes 2 and 3) or SRhpx (lanes 4 and 5). C, splicing of β-globin transcript in NEnor (lane 1) and NEnor (lane 2) or with increasing amounts (0.25–0.75 μg) of SRnor (lanes 3–5) or SRhpx (lanes 6–8). D, average HIF-3α construct splicing efficiencies (from three experiments) quantified by phosphorimaging. E, average IPAS construct splicing efficiencies with SR proteins (from three experiments) quantified by phosphorimaging. F, average β-globin splicing efficiencies with SR proteins (from three experiments) quantified by phosphorimaging. Splicing efficiency in nuclear extracts without additional SR proteins is denoted as C in panels D–F.

**FIGURE 4.** Influence of SRnor and SRhpx on HIF-3α, IPAS, and β-globin pre-mRNAs splicing in hypoxic HeLa cell nuclear extracts. A, splicing of HIF-3α transcript in NEnor (lane 1) and NEhpx (lane 2) or with increasing amounts (0.25–0.5 μg) of SRnor (lanes 3 and 4) or SRhpx (lanes 5 and 6). B, splicing of IPAS transcript in NEhpx (lane 1) or with increasing amounts (0.25–0.5 μg) of SRnor (lanes 2 and 3) or SRhpx (lanes 4 and 5). C, splicing of β-globin transcript in NEhpx (lane 1) and NEhpx (lane 2) or with increasing amounts (0.25–0.75 μg) of SRnor (lanes 3–5) or SRhpx (lanes 6–8). D, average HIF-3α construct splicing efficiencies (from three experiments) quantified by phosphorimaging. E, average IPAS construct splicing efficiencies with SR proteins (from three experiments) quantified by phosphorimaging. F, average β-globin splicing efficiencies with SR proteins (from three experiments) quantified by phosphorimaging. Splicing efficiency in nuclear extracts without additional SR proteins is denoted as C in panels D–F.

SR Proteins Isolated from Hypoxic HeLa Cells Are More Phosphorylated Than Those Isolated from Normoxic Cells—It has been shown that the RS domains of SR proteins are extensively phosphorylated (12). As we found that the steady-state concentrations of SR proteins do not change significantly in hypoxic cells compared with normoxic cells (Fig. 6A), we used Western blot assays to assess the phosphorylation status of SR proteins and to correlate it to their ability to activate alternative HIF-3α splicing patterns. Western blot analysis of SR proteins (isolated from HeLa cells cultured under hypoxic conditions) showed that the RS domain was less efficiently recognized by mAb104 (Fig. 6B). These results suggest that the phosphorylation status of the SR protein RS domain in hypoxic cells might be different compared with normoxic cells. To define the differences in SR protein phosphorylation, we performed a two-dimensional gel analysis of purified SR proteins (29) isolated from normoxic or hypoxic cells (Fig. 6A). Two-dimensional gel Western blot analysis (using an anti-SR antibody) revealed that the isoelectric point of a large fraction of SRhpx was significantly shifted toward the acidic side of the gel (Fig. 6C), indicating that hypoxic SR proteins (isolated from hypoxic cells) are hyperphosphorylated compared with SR proteins from normoxic extracts.

Hypoxic Cells Possess Enhanced SR Protein Kinase Levels—Next we compared SR protein kinase expression levels in normoxic and hypoxic HeLa cells. We found out that expression of CLK1, SRPK1, and SRPK2 was elevated at the mRNA level in hypoxic cells compared with normoxic ones, the most elevated ones being the CLK1 and SRPK1 mRNA levels. SRPK2 mRNA levels were moderately elevated (Fig. 6D). CLK1 kinase is predominantly localized in the nucleus (17). Therefore, we monitored CLK1 protein levels in HeLa cell nuclear extracts, whereas we used whole cell lysates for SRPK1 and SRPK2 pro-

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**Hypoxygenation**

**FIGURE 4.** Influence of SRnor and SRhpx on HIF-3α, IPAS, and β-globin pre-mRNAs splicing in hypoxic HeLa cell nuclear extracts. A, splicing of HIF-3α transcript in NEnor (lane 1) and NEhpx (lane 2) or with increasing amounts (0.25–0.5 μg) of SRnor (lanes 3 and 4) or SRhpx (lanes 5 and 6). B, splicing of IPAS transcript in NEhpx (lane 1) or with increasing amounts (0.25–0.5 μg) of SRnor (lanes 2 and 3) or SRhpx (lanes 4 and 5). C, splicing of β-globin transcript in NEhpx (lane 1) and NEhpx (lane 2) or with increasing amounts (0.25–0.75 μg) of SRnor (lanes 3–5) or SRhpx (lanes 6–8). D, average HIF-3α construct splicing efficiencies (from three experiments) quantified by phosphorimaging. E, average IPAS construct splicing efficiencies with SR proteins (from three experiments) quantified by phosphorimaging. F, average β-globin splicing efficiencies with SR proteins (from three experiments) quantified by phosphorimaging. Splicing efficiency in nuclear extracts without additional SR proteins is denoted as C in panels D–F.

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**Hypoxygenation**

**FIGURE 4.** Influence of SRnor and SRhpx on HIF-3α, IPAS, and β-globin pre-mRNAs splicing in hypoxic HeLa cell nuclear extracts. A, splicing of HIF-3α transcript in NEnor (lane 1) and NEhpx (lane 2) or with increasing amounts (0.25–0.5 μg) of SRnor (lanes 3 and 4) or SRhpx (lanes 5 and 6). B, splicing of IPAS transcript in NEhpx (lane 1) or with increasing amounts (0.25–0.5 μg) of SRnor (lanes 2 and 3) or SRhpx (lanes 4 and 5). C, splicing of β-globin transcript in NEhpx (lane 1) and NEhpx (lane 2) or with increasing amounts (0.25–0.75 μg) of SRnor (lanes 3–5) or SRhpx (lanes 6–8). D, average HIF-3α construct splicing efficiencies (from three experiments) quantified by phosphorimaging. E, average IPAS construct splicing efficiencies with SR proteins (from three experiments) quantified by phosphorimaging. F, average β-globin splicing efficiencies with SR proteins (from three experiments) quantified by phosphorimaging. Splicing efficiency in nuclear extracts without additional SR proteins is denoted as C in panels D–F.

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**Hypoxygenation**
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FIGURE 5. Preincubation of SR
nor in hypoxic nuclear extract results in loss of ability to activate HIF-3α pre-mRNA splicing. A, HIF-3α transcript splicing in NEnor (lane 1, 2, 5, and 6) or NEpox (lane 3, 4, 7, and 8). 0.5 μg of SRnor was added into splicing reactions (lanes 2, 4, 6, and 8). Before the addition of RNA into the splicing reaction, SR proteins were preincubated either in NEnor (lane 6) or in NEpox (lane 8) for 20 min at 30 °C. B, average of HIF-3α construct splicing efficiencies (from three experiments) quantified by scanning. Splicing efficiency in nuclear extracts without additional SR proteins is denoted as C in panel B.

Proteins expression analysis, which are detected both in the cytoplasm and nucleus (22). Expression of CLK1 and SRPK1 was significantly elevated at the protein level in hypoxic cells, whereas SRPK2 protein levels were only very slightly enhanced in hypoxic cells (Fig. 6E).

Hypoxia-induced CLK1 Expression Is HIF-1-dependent—As only CLK kinases are able to phosphorylate the entire RS domain to give a hyperphosphorylated form of SR proteins (17, 22), we performed a bioinformatics analysis of the CLK1 promoter region. The analysis showed that there are five potential HIF binding sites (5'-d(NCGTG)-3'). To investigate if enhanced expression of CLK1 kinase in hypoxic cells is regulated by HIF-1, we used anti-HIF-1α antibodies to perform chromatin immunoprecipitation experiments. Immunoprecipitated DNA was subjected to real-time PCR with primers that amplify a promoter fragment spanning potential HIF binding sites. As shown in Fig. 6F, this analysis showed a 3-fold increase in amplification of the CLK1 promoter region in extracts from hypoxic cells compared with normoxic cells, demonstrating that HIF-1α is recruited to the CLK1 promoter region in a hypoxia-dependent manner.

Reduction of CLK1 Expression or Inhibition of Its Kinase Activity Reduces Hypoxia-dependent Cellular Gene Splicing—CAIX is a hypoxia-induced, cancer-associated carbonic anhydrase isoform with functional involvement in pH control and cell adhesion. It has been reported that two alternatively spliced carbonic anhydrase IX mRNAs, AS CAIX and FL CAIX, are produced from carbonic anhydrase IX pre-mRNA. The human AS CAIX isoform is produced in very low amounts and does not contain exons 8–9. CAIX full-length mRNA production is hypoxia-inducible (32).

Expression of Cyr61 is down-regulated in prostate cancer (33) and leiomyoma (34), and this fact suggests that being under certain circumstances Cyr61 might behave as a tumor suppressor. Two distinct, hypoxia-regulated, alternatively spliced Cyr61 mRNAs are generated in breast cancer cell lines. One isoform contains correctly spliced mRNA (joining all 4 exons or intron skipping (IS) isoform); another isoform between exons 3 and 4 contains intron 3 retention (intron-retaining (IR) isoform) (30, 35) showing that RNA alternative splicing might play an important role in expression regulation of this protein. The retention of intron 3 creates two stop codons within the intronic sequence (30). It is not known if a truncated protein is expressed from the Cyr61 IR mRNA (35).

At first, we monitored the effects of siRNA-mediated down-regulation of CLK1 expression on pre-mRNA splicing of endogenous cell transcripts of well characterized hypoxia target genes CAIX and Cyr61 (Fig. 7A). Slight reduction of AS CAIX isoform was observed in normoxic HeLa cells treated with CLK1 siRNA. Expressions of both CAIX mRNA isoforms are enhanced in hypoxic cells. Reduction of CLK1 levels by specific siRNA treatment almost does not affect AS CAIX mRNA isoform formation and reduces FL CAIX isoform formation in cells (Fig. 7A). Notably, reduction of CLK1 expression decreases not only FL CAIX mRNA but also CAIX cellular protein level (Fig. 7C), indicating that for enhanced expression of CAIX in hypoxic cells increased CLK1 expression is needed.

In the Cyr61 pre-mRNA alternative splicing case in cells, cultivated under hypoxic conditions, formation of Cyr61 IR mRNA isoform is promoted (Fig. 7, A and D, lanes 1 and 3). Such results possibly point to the fact that under certain circumstances Cyr61 mRNA isoform expression pattern changes from IS to IR. Reduction of the CLK1 kinase cellular level increased the cellular IS Cyr61 mRNA formation and reduced the IR mRNA level (Fig. 7, A and D) as in normoxic as in hypoxic cells. HeLa is a tumor cell line; thus it is probably not surprising that the observed Cyr61 pre-mRNA splicing profiles in these cells are more similar to the observed in tumors rather than in healthy tissues (30).
FIGURE 6. Hypoxia induces SR protein hyperphosphorylation by a hypoxia-dependent increase in CLK1 kinase cellular levels. A, SDS-PAGE of SRnor and SRhpx proteins. B, Western blot analysis of SRnor and SRhpx proteins with mAb104 antibody. C, Western blot of two-dimensional gel electrophoresis of SRnor and SRhpx proteins with anti-SR antibody. D, CLK1, SRPK1, and SRPK2 expression at mRNA level in hypoxic and normoxic HeLa cells. E, CLK1, SRPK1, and SRPK2 protein expression in hypoxic and normoxic HeLa cells. F, ChIP to monitor recruitment of HIF-1α to the CLK1 promoter under hypoxic conditions (0–24 h). Antibodies against IgG and HIF-1α were used. Potential HIF-1α binding sites within the CLK1 promoter were amplified using real-time PCR as described under "Materials and Methods." Each bar represents the mean ± S.D. of three to five independent experiments. p < 0.05 comparing 21% versus 1% O2.

FIGURE 7. Reduction of CLK1 protein levels or kinase activity changes hypoxia-dependent CAIX and Cyr61 alternative pre-mRNA splicing. A, siRNA-mediated reduction of CLK1 mRNA expression correlates with reduced formation of FL CAIX mRNA and changed Cyr61 pre-mRNA alternative splicing in hypoxic cells. −, normoxic (nor, lane 1) and hypoxic (lane 3) HeLa cells treated with control siRNA. +, normoxic (lane 2) and hypoxic (lane 4) HeLa cells treated with CLK1 specific siRNA. B, cell treatment with CLK1 kinase inhibitor TG003 correlates with reduced formation of FL CAIX mRNA and changed Cyr61 pre-mRNA alternative splicing in hypoxic cells. −, normoxic (lane 1) and hypoxic (lane 3) HeLa cells untreated with TG003. +, normoxic (lane 2) and hypoxic (lane 4) HeLa cells treated with TG003. C, normoxic or hypoxic cell treatment with CLK1-specific siRNA reduces CLK1 (lanes 2 and 3) and FL CAIX cellular protein levels. −, normoxic (lane 1) and hypoxic (lane 3) HeLa cells treated with control siRNA. +, normoxic (lane 2) and hypoxic (lane 4) HeLa cells treated with CLK1 specific siRNA. D, quantitation of the Cyr61 mRNA IR and IS isoform relative ratio from normoxic (lane 1 and 2) or hypoxic (lanes 3 and 4) cells treated with nonspecific siRNA (lanes 1 and 3) or with CLK1 specific siRNA (lanes 2 and 4). E, quantitation of the Cyr61 mRNA IR and IS isoform relative ratio from normoxic (lane 1 and 2) or hypoxic (lanes 3 and 4) cells untreated with CLK1 inhibitor TG003 (lanes 1 and 3) or treated with TG003 (lanes 2 and 4). 35c and 25c, 35 or 25 PCR cycles were performed.
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For further investigation of the effects of SR protein hyperphosphorylation on hypoxia-dependent splicing, cells cultivated either under normoxic or hypoxic conditions were treated with CLK1 kinase inhibitor TG003 (36). Inhibition of kinase activity revealed that CAIX (Fig. 7B) and Cyr61 (Fig. 7, B and E, lanes 1 and 3) mRNA isoform formation pattern is similar to that observed in cells treated with CLK1 siRNA, i.e. we observed a decrease in FL CAIX isoform formation and a change from IS to IR Cyr61 mRNA formation as in normoxic as in hypoxic cells. The results indicate that CLK1 cellular protein level/activity is an important factor for hypoxia-dependent splicing regulation. Our data demonstrate that an HIF-1-dependent increase in SR protein kinase (CLK1) expression and an increase in its activity in hypoxic HeLa cells is important for regulation of hypoxia-dependent splicing.

Discussion

SR proteins are evolutionary conserved phosphoproteins. They bind to pre-mRNA molecules to promote both constitutive and alternative splicing. Binding sites for SR proteins are not only limited to alternatively spliced exons but have also been verified for exons of constitutively spliced pre-mRNAs (37). Phosphorylation of the RS domain of SR proteins has a great impact on their functionality, as it may affect their binding to target mRNAs, their interaction with other proteins, and their intracellular localization (38).

A number of SR protein kinases have been identified and shown to specifically phosphorylate serine residues within the RS domain of SR proteins. These include SRPK, CLK/STY kinase, cdc2p34, and topoisomerase (37). Hypoxia has long been recognized as a common feature of solid tumors and a negative prognostic factor for response to treatment and survival of cancer patients. Extensive rearrangements in transcription, pre-mRNA splicing, and translation occur in hypoxic cells (39, 40). The biological responses to hypoxia involve induction of transcription of a network of target genes, a process that is coordinately regulated by HIFs (1, 2, 41–43).

Here, for the first time we report that to adapt to hypoxic condition changes, SR protein phosphorylation level is changed in HeLa cells. Also we show that hypoxia-inducible factor HIF-1 is indirectly involved in hypoxia-dependent splicing regulation. An increase in HIF-1-regulated CLK1 gene transcription level causes an increase in CLK1 protein levels in hypoxic cells. This change generates hyperphosphorylated SR proteins. Enhanced SR protein phosphorylation changes their RNA interaction specificity. SR proteins start to interact with RNA sequences with which they do not interact or interact very inefficiently in normoxic cells. It seems that in hypoxia-hyperphosphorylated SR proteins are recruited to pre-mRNA molecules, where they participate in splicing (44) to produce mRNA, from which synthesized products are needed for cell adaptation to changing surrounding conditions.

Detected enhanced CLK and SRPK expression levels in hypoxic HeLa cells indicate that enhanced SR protein kinase activity is required to adapt the cell to reduced oxygen tension and, therefore, to change pre-mRNA splicing. This suggests cooperation between these kinases in hypoxic cells. Reduction of CLK1 protein level or in kinase activity inhibition changes CAIX and Cyr61 pre-mRNA alternative splicing and also reduces CAIX protein levels in the cell, suggesting that the expression level of this kinase plays an important role in hypoxia-dependent alternative pre-mRNA splicing regulation.

In summary, we have reconstituted hypoxia-induced alternative pre-mRNA splicing of the HIF-3 locus and showed that hypoxia alters the activity of SR proteins via an increase in SR protein phosphorylation levels after up-regulation of the expression of SR protein kinases. Under hypoxic conditions, HIF-1 is recruited to HRE motifs within the CLK1 promoter, suggesting that this gene represents a direct hypoxia target gene. Using cellular CAIX and Cyr61 pre-mRNAs as the model system, we showed that CLK1 expression level is important for hypoxia-dependent FL CAIX and Cyr61 alternative pre-mRNA splicing.

Our finding that changes in SR protein activity promote hypoxia-dependent splicing may provide a more general mechanism underlying hypoxia-dependent changes in pre-mRNA splicing. The complexity of the cellular hypoxic response shows that there is a strong reason to assume that the activity and/or function of other splicing factors is altered in hypoxia.

Author Contributions—Y. M., L. P., and A. K. conceived and designed the experiments. E. J. and A. K. designed the constructs, performed experiments, and analyzed the results shown in Figs. 1–5. E. J. and L. V. performed experiments and analyzed the results shown in Figs. 6 and 7. L. P. and A. K. wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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