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Regulation of Alternative Splicing by Reversible Protein Phosphorylation*[S]

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The vast majority of human protein-coding genes are subject to alternative splicing, which allows the generation of more than one protein isoform from a single gene. Cells can change alternative splicing patterns in response to a signal, which creates protein variants with different biological properties. The selection of alternative splice sites is governed by the dynamic formation of protein complexes on the processed pre-mRNA. A unique set of these splicing regulatory proteins assembles on different pre-mRNAs, generating a "splicing" or "messenger ribonucleoprotein code" that determines exon recognition. By influencing protein/protein and protein/RNA interactions, reversible protein phosphorylation modulates the assembly of regulatory proteins on pre-mRNA and therefore contributes to the splicing code. Studies of the serine/arginine-rich protein class of regulators identified different kinases and protein phosphatase 1 as the molecules that control reversible phosphorylation, which controls not only splice site selection, but also the localization of serine/arginine-rich proteins and mRNA export. The involvement of protein phosphatase 1 explains why second messengers like cAMP and ceramide that control the activity of this phosphatase influence alternative splicing. The emerging mechanistic links between splicing regulatory proteins and known signal transduction pathways now allow in detail the understanding how cellular signals modulate gene expression by influencing alternative splicing. This knowledge can be applied to human diseases that are caused by the selection of wrong splice sites.

Regulation of Splice Site Selection

Most protein-coding genes contain introns that are removed in the nucleus by RNA splicing during pre-mRNA processing. Parts of the pre-mRNA can be either included or excluded in the mature mRNA, which is achieved by alternative splicing.

This process is much more widely used than previously thought and was recently estimated to affect between 74 and 88% of human genes (1, 2), but the exact number still needs to be determined. Because most alternative exons encode protein modules, their alternate use allows multiple proteins to be generated from a single gene, which increases the coding potential of the genome. Alternative splicing generates protein isoforms with different biological properties, such as a change in protein/protein interaction, subcellular localization, or catalytic ability (3). More than one quarter of alternative exons introduce premature stop codons in their mRNAs. This can result either in the formation of truncated proteins or in the degradation of the mRNA by nonsense-mediated decay. Recent array analyses indicate that, although frequently found, alternative exons with premature stop codons appear to be generally present only in low abundance, which questions their role as a general shutoff mechanism for protein production (4, 5).

Splice site recognition and pre-mRNA splicing are dynamic processes that involve constant remodeling of proteins and ribonuclear protein particles on the pre-mRNA being processed. They are connected to other processing steps, such as transcription, 5′-end capping, 3′-end polyadenylation, and nuclear export. Splicing regulatory proteins often function in multiple processing events while they remain bound to "their" pre-mRNA (6). The splice sites that flank exons in higher eukaryotes are highly degenerate and are not sufficient for precise exon recognition. Therefore, protein complexes form on the pre-mRNA and facilitate the identification of splice sites with high accuracy. The majority of these splicing regulatory proteins belong to two major classes: hnRNPs[2] and SR proteins. These proteins contain RNA-binding and protein/protein interaction domains. They bind with low specificity to accessible, mostly single-stranded parts of the pre-mRNA (7). To overcome the low RNA binding specificity, splicing regulatory proteins use their protein interaction domains to bind to each other. Again, a low specificity is characteristic for their protein/protein interaction. Once these protein complexes have formed around an exon, they aid ribonuclear protein components of the core spliceosome in establishing RNA/RNA interaction at the 5′-splice site and at the branch point. A well studied interaction occurs between SR proteins and the protein component of U1 snRNP. This interaction is important because the RNA component of the U1 snRNP interacts with the 5′-splice site. The high fidelity of exon recognition is thus achieved by the combination of multiple weak protein/protein, protein/RNA, and RNA/RNA interactions, which are schematically shown in supplemental Fig. 1. Different pre-mRNAs seem to associate with a unique arrangement of proteins, constituting the "splicing" or "mRNP code." By interacting with the spliceosome, the protein complexes forming on the pre-mRNA, *i.e.* the splicing code, determine which RNA parts will be removed as introns

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[2] The abbreviations used are: hnRNP, heterogeneous nuclear ribonucleoprotein; SR protein, serine/arginine-rich protein; snRNP, small nuclear ribonucleoprotein; mRNP, messenger ribonucleoprotein; tra2, transformer-2; PP, protein phosphatase; PK, protein kinase; TNFα, tumor necrosis factor-α.

and which parts will be included in the mature mRNA (8). The low affinity of each interaction is an intrinsic property of pre-mRNA processing. It allows proteins to bind and detach from pre-mRNA during the splicing process. These dynamic rearrangements would be impossible if the proteins bind tightly to RNA or to each other. It is also important that regulatory proteins have the ability to recognize degenerate RNA sequences because the low sequence specificity allows exon recognition without interfering with the protein coding requirements of the mRNA. The low affinity of individual interactions also enables splicing factors to work in a concentration-dependent manner, as they can compete for common binding sites, which can be either RNA sequences or protein interaction domains. As a result, the inclusion of an alternative exon frequently depends on the relative concentration of regulatory factors, which can have antagonistic activities (9, 10).

Mass spectroscopic analyses of splicing complexes have identified ~300 proteins associated with splicing complexes (reviewed in Ref. 11). Because these proteins undergo alternative splicing, the actual number of regulatory proteins might be much higher. Whereas some proteins, such as NOVA (12), are highly tissue-specific, most splicing regulatory proteins are expressed in all tissues, although their expression can vary between cell types within a tissue (13). In contrast, numerous alternative exons show tissue or cell type-specific expression. This raises the intriguing question of how the combination of ubiquitous factors can lead to a cell type-specific readout. One possibility would be that different expression levels of ubiquitous splicing factors with opposing effects on exon usage result in cell type-specific alternative splicing. However, this model cannot explain how cells can rapidly change splice site selection in response to a stimulus (14). Because splicing is coupled to transcription, signals changing transcription rates or chromatin structure can influence splice site selection (reviewed in Ref. 15). Most splicing factors are post-translationally modified by phosphorylation and also by glycosylation (16) or methylation (17). Here, I review reports showing that the reversible phosphorylation of splicing regulatory proteins influences splice site selection. These findings indicate that phosphorylation is an important contributor to the splicing code that governs the fate of a pre-mRNA.

Reversible Phosphorylation Regulates Protein Interaction and Intracellular Localization of Splicing Factors

Changes in splicing were observed after various cellular signals, such as cellular stress, receptor activation, and temperature change (Refs. 18–20; reviewed in Refs. 14, 21, and 22). For example, tra2-β1 isoforms change within 1 h in animal hippocampi after increased neuronal activity (23), a timeframe also seen in other systems (14). A change in splice site selection can be observed only after existing isoforms are degraded and replaced with newly processed pre-mRNA. The alteration in splice site selection will therefore occur in $<$1 h. Studies in several systems demonstrated that this rapid cellular process does not require protein synthesis and is often concomitant with a change in phosphorylation of splicing factors (14). It is therefore not surprising that a change in alternative splice site selection was observed when the activity of kinases or phosphatases acting on splicing factors was manipulated (24–26). These phosphorylation changes are reversible, as early studies demonstrated that complete inhibition of phosphatases or absence of ATP completely blocked the splicing reaction (27–29).

Phosphorylation alters the interaction between individual splicing regulatory proteins and between some of these proteins and RNA. One of the consequences of phosphorylation is a difference in the mRNP composition that alters splice site selection. The other consequence is a different subcellular localization of regulatory proteins, which alters their concentration in areas where splicing occurs and again results in a change in splice site selection.

Change in mRNP Formation—Among the best studied classes of splicing regulatory proteins are SR proteins and hnRNPs. SR proteins contain one or two RNA recognition motifs and a characteristic RS domain that is rich in serine and arginine residues. This domain can interact with both other proteins and RNA (30, 31), and its serine residues are subject to reversible phosphorylation. A change in phosphorylation changes its ability to interact with other proteins. For example, phosphorylation of SRp38 decreases its binding to U1 70-kDa snRNP, but increases its binding to tra2-α (19). Phosphorylation of the SR protein SF2/ASF increases its binding to U1 70-kDa snRNP (32) and decreases its binding to the RNA export factor TAP/NXF1 (33). Finally, the ability of the RS domain to bind to RNA depends on its phosphorylation (31, 32). Phosphorylation-dependent interactions were also described for hnRNPs, a structural diverse group of proteins that have been operationally defined as proteins that associate with nascent transcripts (34). They contain different RNA-binding and protein interaction domains. Phosphorylation of TIA-1 by the Fas-activated kinase FAST promotes its interaction with U1 snRNP C (35), and phosphorylation of PP2Cγ increases its interaction with hnRNP YB-1. In both cases, splice site selection is dependent on phosphorylation.

Changes in Intracellular Localization—Several splicing factors shuttle between the nucleus and the cytosol. This process is sensitive to reversible phosphorylation that mediates interactions with export and import systems (36). For example, binding of the shuttling SR proteins 9G8 and SF2/ASF to the nuclear export factor TAP/NFX1 is increased by dephosphorylation (33), and predominantly the hypophosphorylated forms of the proteins accumulate in the cytosol (37). Analysis of hnRNP A1 and p62/SAM68 demonstrated that the phosphorylation sites are near or within nuclear export/import signals, which explains their dependence on phosphorylation (38, 39). The cellular localization of hnRNP A1 is regulated by serine phosphorylation of the "F-peptide," which spans the 18 amino acids adjacent to the M9 nuclear motif that is important for both nuclear import and export. hnRNP A1 shuttles rapidly between the nucleus and the cytosol, from which it is imported by the transport receptor transportin-1. Serine phosphorylation of the F-peptide reduces binding between hnRNP A1 and transportin-1, which explains why this phosphorylation leads to hnRNP A1 accumulation in the cytosol (38). Osmotic shock activates the MAPK3/6 (mitogen-activated protein kinase)-p38 signal-

ing pathway, which in turn results in phosphorylation of the F-peptide and accumulation of hnRNP A1 in the cytosol (40).

The decrease in hnRNP A1 nuclear concentration leads subsequently to a change in splice site selection (20). A similar effect of phosphorylation-dependent cellular sequestration on alternative splice site selection has been observed in other systems. For example, brain ischemia that occurs during a stroke causes hyperphosphorylation of the SR-like protein tra2-β1. The hyperphosphorylated protein accumulates in the cytosol, and again, alternative splicing patterns are changed (41). It is not clear why hyperphosphorylated tra2-β1 accumulates in the cytosol, but the affinity of tra2-β1 for the nuclear receptor transportin-SR2 is increased by phosphorylation (42), which could block the release of the protein from the receptor.

Intranuclear Localization—The localization of regulatory proteins within the nucleus is regulated by phosphorylation as well. Splicing factors accumulate in nuclear sites, the "splicing factor compartments" or speckles. From there, they can be released to adjacent areas, where splicing takes place. Hyperphosphorylation of SR proteins by a group of Cdc2-like kinases causes their release from speckles, which results in a change in splice site selection (24, 43). A similar dependence was observed for hnRNPs, where tyrosine phosphorylation changed the intranuclear localization of the splicing factor YT521-B. Src kinase-mediated phosphorylation causes a redistribution of the factor throughout the nucleus, causing a change in splicing of reporter constructs (44).

SR Proteins as an Example for Phosphorylation by Multiple Kinases in Different Cellular Compartments

Phosphorylation of SR proteins and their prototypical founding protein, SF2/ASF, is mechanistically and functionally best understood. Phosphorylation of SF2/ASF is a dynamic process that takes place in different cellular compartments (Fig. 1). The phosphorylation begins in the cytosol with SR protein kinases SRPK1 and SRPK2 phosphorylating the RS domain of SF2/ASF. SRPK1 binds to SF2/ASF and phosphorylates serine residues via a processive mechanism. Surprisingly, the enzyme identifies a docking motif in the seemingly uniform RS domain, which is used to start the phosphorylation (45). Phosphorylation of SF2/ASF facilitates its nuclear import (46), which results in the accumulation of SF2/ASF in nuclear speckles. There, a family of Cdc2-like nuclear kinases acts on already phosphorylated SR proteins. This kinase family has four members, Sty/Clk1 and Clk2–4, which have slightly different expression patterns in tissues. Similar to SR proteins, the Clk kinases contain an RS domain to facilitate protein/protein interactions. Clk kinase action hyperphosphorylates SR proteins, which causes their release from speckles to areas where the splicing reaction takes place (47). Because Clk kinases increase the active concentration of regulatory proteins in areas of splicing, their activity changes alternative splicing patterns (24, 26).

Detailed studies of physiological regulation paradigms identified several other kinases that subsequently act on already phosphorylated SR proteins and fine-tune their function in alternative splice site selection. Glycogen synthase kinase-3 phosphorylates the SR protein SC35, resulting in increased skipping of Tau exon 10 (48). The usage of this exon is misregulated in neurological diseases. Activation of the insulin receptor

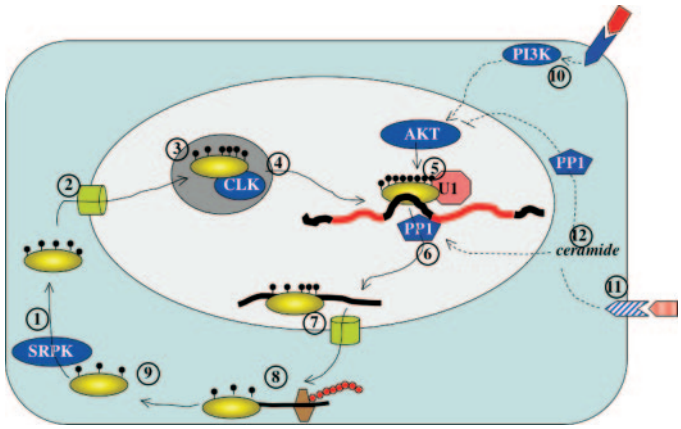

FIGURE 1. **Reversible phosphorylation of SF2/ASF.** SF2/ASF is indicated by *yellow ovals*. Its serine phosphorylation sites are shown as *small black circles*. Kinases acting on SF2/ASF are shown as *blue ovals*. The phosphorylation events are shown in a hypothetical cell, indicated by the *box* with *rounded corners*. Its nucleus is indicated in *light gray*; nuclear speckles are indicated in *dark gray*. SF2/ASF exists in several phosphorylation states that are not clearly defined. "Hyperphosphorylated" or "hypophosphorylated" indicates an increase or decrease, respectively, in phosphorylation relative to a previous state (*step 1*). In the cytosol, SRPK1 and SRPK2 phosphorylate SF2/ASF on its SR domain, which facilitates nuclear import (*step 2*) via a specific transportin-SR transporter, indicated as a *green cylinder*. In the nucleus, this hypophosphorylated form of SF2/ASF accumulates in nuclear speckles (*step 3*), where it is hyperphosphorylated by Clk1–4 kinases (*step 4*), releasing hyperphosphorylated SF2/ASF from speckles to nuclear sites where splicing is occurring (*step 5*). The phosphorylation state of SF2/ASF regulates the interaction with other proteins forming mRNP complexes; for example, it strengthens binding of U1 snRNP to SF2/ASF (*step 5*). The proteins form mRNP complexes with pre-mRNA, indicated as a *line*. Exons are indicated in *black*, and introns in *red*. During the splicing reaction, SF2/ASF is phosphorylated by unknown kinases and dephosphorylated by PP1 (*step 6*). After completion of the splicing reaction, SF2/ASF is dephosphorylated by PP1, which facilitates it nuclear export (*step 7*). Hypophosphorylated SF2/ASF remains associated with some mRNAs and helps in their translation (*step 8*). The nascent protein is shown as a string of *red beads*, and the ribosome in *brown*. Hypophosphorylated SF2/ASF is released for the next round of processing (*step 9*). Known signal transduction pathways regulate these phosphorylation events. Activation of the phosphatidylinositol 3-kinase (*PI3K*) pathways via growth hormone or insulin receptors leads to stimulation of Akt, which phosphorylates SF2/ASF (*step 10*). It is not clear whether this phosphorylation occurs in the cytosol or in the nucleus as shown in the figure. TNFα elevates cellular ceramide levels (*step 11*), activating PP1, which dephosphorylates both SF2/ASF and Akt (*step 12*).

activates PKB (Akt2), which phosphorylates the SR proteins SRp40, SF2/ASF, and 9G8, which changes alternative splicing of PKC and fibronectin mRNA (49, 50). PKAα and PKAβ, which are activated by an increase in cAMP concentration, phosphorylate SR proteins *in vitro* and change splice site selection *in vivo* (51). Finally, topoisomerase I that is overexpressed in certain cancer cells phosphorylates SR proteins, which alters alternative splicing (52).

These examples demonstrate that SR proteins are targeted by several kinases that are regulated by different signal transduction pathways. Although RS domains look uniform, kinases can identify distinct regions for phosphorylation. Phosphorylation of SR proteins takes place in different cellular compartments, which reflects multiple functions of SR proteins.

Phosphatases Acting on Splicing Regulatory Proteins

In contrast to the estimated 474–518 protein kinases that represent 1.7% of human proteins (53), only ~25 serine/threonine protein phosphatases are known (54). The low number of protein phosphatases is the result of combinatorial control, where

the protein phosphatase represents only the catalytically active subunit that forms numerous regulatory complexes with other proteins. PP1 and PP2Cγ have been identified to dephosphorylate splicing regulatory proteins. Complete blocking of phosphatases inhibits splicing, as dephosphorylation is necessary for the transesterification step (55, 56). However, modulation of phosphatase activity *in vitro* (57) and *in vivo* (58, 59) influences alternative exon usage, demonstrating that adjusting phosphatase activity can be used by cells to control alternative splicing.

Regulation by PP1—Three human isogenes (PP1α, -β, and -γ) encode catalytic subunits for PP1. These subunits bind to a degenerate RV*X*F motif present in their interacting proteins. Binding to these regulatory subunits is regulated by phosphorylation (54, 60). Several proteins, including inhibitor-1, inhibitor-2, DARPP-32, and NIPP1 (nuclear inhibitor of PP1), that block PP1 activity through tight binding have been identified (54, 61).

The cellular localization of PP1α, -β, and -γ depends on their binding to interacting proteins and is changed by cell activity. PP1 therefore shuttles between different cellular compartments (62). High-throughput screens identified numerous nuclear proteins, including many hnRNPs, as binding partners for PP1γ (63). The PP1-binding motif RV*X*F is phylogenetically conserved in the SR proteins SF2/ASF, SRp30c, and tra2-β1, which bind to PP1 depending on the presence of this motif, which explains why PP1 dephosphorylates SR proteins (64).[3] Mutation of this motif in SF2/ASF, SRp30c, and tra2-β1 abolishes the ability of these proteins to change splice site selection *in vivo*.[3] These data indicate that PP1 binds directly to some splicing regulatory proteins, which allows direct dephosphorylation of splicing complexes. PP1 can therefore influence alternative splicing events that are co-regulated by SF2/ASF, tra2-β1, and SRp30c. An interesting aspect of PP1 catalytic units is their dynamic localization that depends on the localization of regulatory proteins. A sequestration of PP1, for example, by the well studied activation of the glycogen-targeting subunit (65), would impinge on splice site selection by shifting active PP1 from the nucleus to the cytosol. It is therefore possible that PP1 integrates numerous cellular events with pre-mRNA processing by regulating the phosphorylation state of SR proteins.

Regulation by PP2Cγ—The other well studied phosphatase implicated in alternative splicing is PP2Cγ. It was identified in a splicing complementation assay to promote spliceosome assembly and is associated with purified spliceosomes. PP2Cγ binds to hnRNP YB-1. This binding is strengthened by phosphorylation and, interestingly, is antagonized by autodephosphorylation of PP2Cγ. PP2Cγ therefore regulates YB-1-dependent splicing events *in vivo* (25, 66).

Reversible Phosphorylation Links Pre-mRNA Processing to Signal Transduction Pathways

It is well established that alternative splice site selection can be rapidly changed according to signals that cells receive (recently reviewed in Refs. 14, 21, and 22). Reversible phosphorylation then links some of these signals to the spliceosome. For example, it was shown previously that insulin activates PKB/Akt, which phosphorylates the SR proteins SRp40, SF2/ASF, and 9G8 (58). These phosphorylation events alter the splicing pattern of PKCβ toward exon inclusion, which creates a PKCβ isoform that facilitates glucose uptake. TNFα is a signaling molecule that is elevated in people with insulin resistance, but its molecular link to insulin resistance is not clear. One of the effects of TNFα is an increase in ceramide, a sphingolipid second messenger. Ceramide activates allosterically PP1, which directly dephosphorylates SR proteins. One of the SR proteins that is dephosphorylated in response to TNFα treatment is SRp40. Dephosphorylation of SRp40 then changes the splicing pattern of PKCβII toward exon skipping, which creates a PKCβ isoform that does not stimulate glucose uptake (58). This system demonstrates nicely how different signals can cause a physiologically meaningful switch of exon usage.

Reversible Phosphorylation and Disease

Wrong splice site usage has been observed in numerous diseases. Missplicing of cellular genes can be either a symptom of an underlying molecular defect or the actual cause of the disease. Changes in alternative splicing are frequently observed in cancer, where they are probably the result of cellular transformation. In several genetic diseases, such as FTDP-17 and spinal muscular atrophy, a change in splicing is caused by mutations and is the actual cause of the disease (recently reviewed in Ref. 67). Furthermore, regulated alternative splicing events control apoptosis and are necessary for replication of many viruses, such as human immunodeficiency virus. Influencing alternative splicing pathways would be one way to treat such diseases, to send undesired cells into apoptosis, or to stop viral replication.

Overexpression of kinases (24) or an increase in splicing factors through viral delivery (68) changes splicing into a desired outcome, *i.e.* could reverse the wrong splice site selection observed in a disease. This proves the principle that splicing defects can be treated by manipulation of phosphorylation events. Chemical screens identified inhibitors of SR protein kinases that reduce Sindbis virus replication in cell systems (69), and topoisomerase I inhibitors are in use for chemotherapy. In both cases, alternative exon usage is changed. However, so far, a change in phosphorylation of splicing factors changes the usage of numerous exons. The challenge for further drug development will be to manipulate signal transduction pathways to specifically influence a singular splicing event.

Conclusion

It is now clear that reversible phosphorylation has a strong impact on pre-mRNA processing. The reversible phosphorylation of splicing factors occurs in different cellular compartments. It is achieved by different kinases and phosphatases, allowing the convergence of their regulatory signals on splice site selection. Regulatory proteins like PP1 move between cellular compartments, depending on the activity of the cell. Because of this dynamic behavior, splice site control will be subject to indirect regulation by sequestration of regulatory phosphatases and kinases, which links splicing to other activities of the cell.

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