RESEARCH COMMUNICATION

Alternative processing of the sarco/endoplasmic reticulum Ca^{2+} -ATPase transcripts during muscle differentiation is a specifically regulated process

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Expression of the muscle-specific 2a isoform of the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA2) requires activation of an otherwise inefficient splice process at the 3'-end of the primary gene transcript. We provide evidence that SERCA2 splicing is a specifically regulated process, rather than the result of an increase in general splice efficiency or a decrease in polyadenylation efficiency at the 5'-most polyadenylation site.

This is indicated by the fact that changes in general splice and polyadenylation efficiency, as observed during B-cell maturation, did not affect SERCA2 splicing. Furthermore, expression and overexpression studies did not support the hypothesis that changes in the level of the alternative splice factor ASF/SF2 or other arginine and serine rich proteins are sufficient to obtain the regulation of muscle- and neuronal-specific splicing.

INTRODUCTION

The Ca^{2+} -transport ATPases of the sarcoplasmic or endoplasmic reticulum (SERCAs) mediate the uptake of Ca^{2+} into intracellular stores, such as the sarcoplasmic or endoplasmic reticulum (for a review see [1]). They are encoded by three different SERCA genes and additional alternative processing of the primary gene transcripts leads to five different protein isoforms [2–5]. These isoenzymes are expressed in a tissue-specific and developmentally regulated way. Tissue-specific alternative 3'-end processing of the SERCA2 mRNA gives rise to two distinct protein isoforms which differ in their C-terminal part. This divergence is responsible for functional differences between SERCA2a and SERCA2b [6,7]. These isoforms show a tissue-specific expression pattern: SERCA2b is found in non-muscle tissues and in smooth muscle, whereas SERCA2a is typical of heart, slow-twitch skeletal muscle and is also present in smooth muscle.

The two different SERCA2 isoforms are translated from four different mRNAs (class-1 to class-4) generated by tissue-dependent alternative processing of the SERCA2 transcript. The class-1 mRNA is formed in cardiac muscle, slow-twitch skeletal muscle and smooth muscle by a splicing process, which removes an optional intron at the 3'-end of the SERCA2 messenger. The class-2 mRNA is found in non-muscle cells and is polyadenylated in between the muscle-specific 5' donor and 3' acceptor splice sites. The class-3 mRNA is a minor transcript which is found in all tissues. It contains the muscle-specific intron and it is polyadenylated at a downstream site. Finally, the brain-specific class-4 mRNA is formed by a splicing event using a second 5' donor splice site downstream of the muscle donor site and the same 3' acceptor site as in class-1 mRNA. Class-1 encodes the muscle-specific SERCA2a isoform, while class-2, -3 and -4 all encode the non-muscle SERCA2b isoform.

We have studied the alternative processing of the SERCA2 gene transcript using a minigene containing the 3'-end of the SERCA2 gene [8–10]. SERCA2 transcript processing was studied by stably transfecting the minigene in a myogenic cell line

(BC₃H1) that is well characterized with respect to differentiation-dependent SERCA2 processing [11,12]. The effect of different modifications on differentiation-dependent transcript processing was thereby evaluated. These studies demonstrated that: (1) muscle-type splicing is inefficient in non-muscle cells; (2) several features of the mRNA contribute to this inefficiency of splicing in non-muscle cells (these include weak 5' splice sites, a weak polyadenylation site and the presence of a long terminal intron); and (3) SERCA2 splicing must be activated during myogenic differentiation.

The present study concentrates on the question of whether muscle-specific splicing is regulated by changes in the level or activity of general splice factors or, alternatively, is controlled by specific *trans*-acting factors. We therefore evaluated the effect of changes in general processing capacities on alternative SERCA2 splicing by expressing the SERCA2 minigene in a cell system which has previously been characterized in this respect (the B-cell model, [13]). Moreover, we have determined the concentration of arginine and serine rich proteins (SR proteins) and tested whether the alternative splice factor ASF/SF2 can induce SERCA2 splicing or has an effect on the choice of the SERCA2 donor splice site as has been reported for other systems [14–16].

MATERIALS AND METHODS

Construction of the minigene containing the SERCA2 3'-end, as well as the different modifications, have been described in previous papers [8,9].

BC₃H1 mouse myoblasts [American Type Culture Collection (ATCC); CRL1443] were cultured as described previously [8]. HeLa S3 cells (ATCC; CCL2.2) were grown in nutrient mixture Ham's F12 supplemented with 10% (v/v) fetal calf serum, 3.5 mM L-glutamine, 85 µg/ml streptomycin and 85 units of penicillin.

For the transfection of HeLa cells, 5 µg of minigene and 1 µg of the expression vector containing the human ASF/SF2 cDNA

Abbreviations used: 5'D1, 5' donor splice site 1; SERCA, sarco/endoplasmic reticulum Ca^{2+} -ATPase; SR proteins, arginine and serine rich proteins; RT, reverse transcriptase.

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(pCG-ASF), or without the cDNA (pCG), were added to a 75 cm² cell-culture flask with 60–75 % confluent HeLa cells in the presence of 20 µg of lipofectamin (Gibco-BRL, Gaithersburg, MD, U.S.A.). Cells were harvested after 48 h for the transient transfections.

The M12 B-cell line was maintained in RPMI 1640 medium supplemented with 10 % (v/v) fetal bovine serum and 50 µM 2-mercaptoethanol. The S194 plasmacytoma cell line was maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) horse serum. The DEAE-dextran transfection protocol [17] was used for transient expression assays.

Total RNA was isolated from transfected cells and reverse transcriptase (RT) PCR was performed as described previously [9]. ASF/SF2 mRNA detection was performed as described by Cáceres et al. [15].

Nuclear extracts were prepared according to the small-scale procedure described in [18]. Nuclear extract (15 µg) was separated on a 5 mm thick Laemmli-type polyacrylamide gel [12.5 % (w/w) acrylamide]. The proteins were blotted using the semi-dry electroblotting method on Immobilon-P transfer membranes (Millipore, Bedford, MA, U.S.A.). The blots were blocked for 1 h in PBS containing 3 % (v/v) BSA. Antibody staining was by a 16 h incubation at 4 °C in the presence of the primary antibody at the appropriate dilution. This was followed by successive 1 h incubations at room temperature with rabbit anti-mouse IgG + IgM (1 mg/ml, Jackson Immuno Research) diluted 1:2000 and with ¹²⁵I-Protein A (Amersham International, U.K.) diluted 1:1000. Each dilution was performed in PBS + Tween 0.1 % (v/v) and the incubations were followed by a wash in this buffer. The blots were stained with the monoclonal antibody mAb104, which recognizes the family of SR proteins [19,20], and with polyclonal antibodies raised against the last 14 amino acids of the different SR splice factors: anti-ASF, anti-9G8 and anti-SC35 [21,22].

RESULTS

Is SERCA2 splicing regulated by a specific or by a general mechanism?

Two different hypotheses can be formulated to explain the up-regulation of SERCA2 splicing during muscle differentiation. The first hypothesis invokes a specific *trans*-acting factor binding to a *cis*-acting sequence element. A specific splice factor would bind to this element, thereby increasing or decreasing the probability of splicing. The second hypothesis postulates that differential regulation depends on the modulation of the activity of general splice and/or polyadenylation factors, as demonstrated for the regulation of Igµ processing during B-cell maturation [13,23–26]. The 3'-end of the SERCA2 gene structurally resembles that of the Igµ gene in terms of the relative positions of the optional splice and polyadenylation sites. Moreover, two artificial genes, engineered to have a comparable structure to the Igµ gene, were processed similarly when transfected in B-cells or plasma cells. We were therefore interested in whether the general factors responsible for this differential processing would also act on SERCA2.

The SERCA2 minigene was transiently transfected in the M12 and S194 cell lines and RNA was isolated. Oligo(dT)-primed first-strand cDNA was prepared and fragments of the different pCM/βSERCA2 transcripts were PCR amplified. The relative position of the primers used to discriminate between class-1 and class-2/3 RNA is indicated in Figure 1(A). The U1 sense oligonucleotide located in exon 2 of β-globin, combined with the C2 antisense primer located in exon 22 of SERCA2, can amplify two fragments: one of 414 nt, corresponding to class-2/3, and a

Figure 1 Expression of SERCA2 minigene constructs in the mouse B-cell line M12 (B) and in the mouse plasmacytoma cell line S194 (PC)

(A) The pCM/βSERCA2 minigene as well as the location of the different modifications is shown schematically. The processing signals present in SERCA2 are indicated as follows: 5'D1 and 5'D2, 5' donor splice sites 1 and 2 respectively; 3'A, 3' acceptor splice site; pA_u and pA_d, upstream (u) and downstream (d) polyadenylation signals. The relative positions of the sense and antisense PCR primers used to amplify the different RNA classes are depicted as arrows above the sequence. The β-globin regions are shaded and the SERCA2 sequences are open boxes. CMV-IE, cytomegalovirus-immediate early promoter; DRF, downstream flanking region. Mut1 contains an optimized 5'D1 donor site and in the Mut1,Del1 construct the optimization of the 5'D1 is combined with a deletion of 1924 nt in the muscle-specific intron. In Mut4, the upstream polyadenylation signal is deleted. (B) Processing of the pCM/βSERCA2 transcripts in M12 and S194 cells. After transient transfection, cytoplasmic RNA was prepared and was analysed by RT-PCR using the U1, P2 and C2 primers. The amplified fragments as well as the primers are shown on the left of the gel. Lanes M1 and M2 contain molecular-mass markers and in lane C the cDNA sample is replaced by water; nucleotide lengths are indicated on the right. WT, wild type pCM/βSERCA2 construct.

second one of 987 nt that is amplified from a precursor of class-2/3 that still contains the second β-globin intron between exons 2 and 3. The same U1 primer, combined with the P2 antisense oligonucleotide located in exon 25 of SERCA2, can amplify a 617 nt fragment that corresponds to the muscle-specific class-1 mRNA.

We first transfected the wild-type SERCA2 minigene in the mouse B-cell line M12 (producing roughly equal amounts of spliced and polyadenylated Igµ) and in the mouse plasmacytoma cell line S194 (producing mostly cleaved-polyadenylated Igµ). Only the polyadenylated class-2/3 transcripts could be detected in both cell lines (Figure 1B, lanes 1 and 2). There was no apparent activation of splicing in the B-cell line, and this implies that the SERCA2 mRNA is processed differently from other transcripts having a similar structure. These are spliced relatively more in B-cells, whereas polyadenylation is the predominant pattern in the plasmacytoma cells [13]. A remarkable observation is that the precursor containing the β-globin intron is present in B-cells but is completely absent from plasma cells.

We also artificially changed the strength of the muscle-specific 5' donor splice site (5'D1) by introducing mutations to make it more similar to the consensus donor splice site (Figure 1A). This mutation increases the likelihood of splicing in both 10T1/2 and undifferentiated BC₃H1 cells [8,9]. In B-cells and in plasma cells, a class-1 band could be observed. The class-1 band was very faint

Figure 2 Western blots of nuclear extracts prepared from undifferentiated (U) and differentiated (D) BC₃H1 cells

Nuclear extracts were separated on a Laemmli-type polyacrylamide gel [12.5% (w/w) acrylamide]. The proteins were blotted using the semi-dry electroblotting method on Immobilon-P transfer membranes. The blots were stained with the monoclonal antibody mAb104 that recognizes the family of SR proteins (A) and with polyclonal antibodies raised against the last 14 amino acids of different SR splice factors: anti-ASF/SF2 (B), anti-9G8 (C) and anti-SC35 (D).

in B-cells and a more prominent band was observed in plasma cells (Figure 1B, lanes 3 and 4). This indicates that the SERCA2 transcript containing the optimized 5'D1 is spliced more efficiently in plasma cells compared with B cells. This observation again indicates that the regulation of SERCA2 processing is different from Ig μ processing. The induction of splicing is more pronounced when an intron deletion is combined with an optimized 5'D1 splice site (Figure 1B, lanes 5 and 6), and in this case no difference between both cell lines could be observed. Splicing is nearly maximally activated as the class-2/3 band becomes very faint.

We also deleted the upstream polyadenylation site (pA_n) and this did not stimulate muscle-type splicing in either cell type (Figure 1B, lanes 7 and 8). This confirms that a model proposing a competition between splicing and polyadenylation cannot explain the regulation of SERCA2, which is consistent with the conclusion reached using 10T1/2 cells [8].

Are SR proteins involved in regulated SERCA2 splicing?

SR proteins are a family of proteins that play an essential role in constitutive splicing. In addition, some members are also involved in the regulation of alternative splicing. In order to find out whether SR proteins play a role in differentiation-dependent SERCA2 splicing, we first determined which SR proteins are present in BC₃H1 cells and whether there is a differentiation-dependent change in their expression level. This was done by Western blotting, starting from nuclear extracts prepared from undifferentiated and differentiated cells. Using the non-discriminating mAb104 antibody [20], we could only detect one band that was identified as a 35 kDa band using specific antibodies (Figure 2A). These antibodies were specific for three 35 kDa SR proteins (ASF/SF2, SC-35 and 9G8) and stained at the same level (Figures 2B, 2C and 2D). No large differences in expression

Figure 3 The effect of ASF/SF2 overexpression on SERCA2 pre-mRNA processing

Different pCM β /SERCA2 constructs (WT, wild type; Mut1, 5'D1 optimized; Del1, intron deletion 1) were cotransfected in HeLa cells in combination with an expression vector containing the human ASF/SF2 cDNA (pCG-ASF). As a control (C), the expression vector without the cDNA (pCG) was cotransfected. RNA was obtained and RT-PCR was performed. Representations of the amplified fragments and the primers are shown on the left of the gels. (A) Class-1 detection. The amplified fragments represent the muscle-type RNAs. (B) Class-2/3 detection. The amplified fragments represent the non-muscle type mRNAs. (C) Class-4 detection. As a positive control, mRNA obtained from pig cerebellum (Cer) was used. (D) Detection of endogenous and exogenous ASF/SF2 mRNAs using two different forward primers (Fw1 and Fw2) with one reverse primer (R) as described by Caceres et al. [15]. CMV, cytomegalovirus promoter present in the transfected pCG-ASF construct; 5'UTR, 5' untranslated region of the endogenous ASF/SF2. Lane 8 of (A), (B) and (D) contains molecular-mass markers (III and m) with the nucleotide length of the different fragments indicated on the right.

levels could be detected between undifferentiated and differentiated BC₃H1 cells, indicating that variations in the levels of SR proteins do not coincide with the induction of muscle-type splicing in BC₃H1 cells.

That SR proteins are not critically involved in the induction of muscle-specific splicing was confirmed by overexpressing ASF/SF2. ASF/SF2 is the best characterized member of the SR family. We studied the effect of its overexpression on the processing of SERCA2 transcripts. An expression vector containing the cDNA of human ASF/SF2 was cotransfected with the wild-type SERCA2 minigene and two modified constructs (Mut1 and Del1) in HeLa cells. This cell line was chosen as a cell culture model in view of the known effects of ASF/SF2 overexpression on alternative splicing of cotransfected genes [15].

When a wild-type pCM β /SERCA2 was transfected into HeLa cells, no class-1 splicing could be detected. Muscle-type splicing could only be observed when the minigene with the optimized 5'D1 (Mut1) or with the shortened intron (Del1) was transfected. Cotransfecting the wild-type construct with the pCG-ASF expression vector did not result in induction of muscle-type splicing

(Figure 3A, lanes 1 and 2). Furthermore, no additional up-regulation of splicing could be observed when Mut1 or Del1 was cotransfected with the ASF/SF2 expression vector (Figure 3A, lanes 3–6). The ASF/SF2 expression levels were checked at the mRNA level by RT-PCR as described by Caceres et al. [15]. Figure 3(D) shows that in each cotransfection experiment good levels of exogenous ASF/SF2 were obtained. That the overexpression of ASF/SF2 resulted in active protein was demonstrated by co-transfecting the ASF expression construct and an expression vector containing the β -thalassemia gene. This resulted in the expected change in splice pattern of the β -thalassemia transcript (results not shown). The expression levels of the SERCA2 minigene were estimated by RT-PCR for the class-2/3 messenger (Figure 3B).

It has been reported that, both *in vitro* and *in vivo*, ASF/SF2 can also affect the selection of the 5' donor splice site [14,15]. It can act as an alternative splice factor by promoting the choice of the 5' splice site most proximal to the acceptor site. A second donor splice site (5'D2), which is used in neuronal class-4 splicing, is present at the 3'-end of SERCA2 and is more proximal to the acceptor splice site than the muscle-type 5'D1. Therefore we also investigated whether ASF/SF2 overexpression could induce class-4 splicing. As shown in Figure 3(C), this is not the case, since class-4 mRNA could not be detected.

DISCUSSION

A simple hypothesis to explain the alternative splicing at the 3'-end of SERCA2, is that its regulation is based on the organization and relative strength of the different processing signals. This kind of regulation has been proposed for other genes [13,27,28]. We concluded from a previous study that the relative strength and the spatial organization of the different processing signals are indeed necessary prerequisites to obtain regulated splicing [9]. In this study, we wanted to find out whether these prerequisites are sufficient to achieve regulation. Therefore we have studied the processing of SERCA2 during B-cell maturation. Indeed the 3'-end of SERCA2 and of *Ig μ* have a comparable gene architecture and hence similar mRNA processing options. *Ig μ* RNAs are differentially processed in B-cells compared with plasma cells, which has been attributed to competition between splicing and polyadenylation and to global changes in the efficiency of one or both processes during B-cell maturation [13,23–26]. Moreover, it has been shown that other non-related transcripts with a similar structure are processed analogously to the *Ig μ* construct. Despite the remarkable resemblance to the structure of *Ig μ* , SERCA2 transcripts were not differentially processed in B-cells and plasmocytes. This indicates that the documented differences in splicing/polyadenylation capacity of B-cells compared with plasma cells [25] do not affect SERCA2 processing. It also suggests that activation of muscle-type splicing during myogenic differentiation requires the expression and/or activation of a specific *trans*-acting factor that increases splicing efficiency. Such a factor could either bind to a stimulatory *cis*-element (splice enhancer) or it could compete out a non-muscle factor which represses splicing in non-muscle cells. Additional experiments are required to discriminate between both possibilities. Another argument for specific regulation is the fact that, apart from the myogenic cell lines (BC₃H1 and C2C12) and 10T1/2 cells transfected with a myogenin expression vector, no other cell lines were found which processed the transcript of the unmodified pCM β SERCA2 construct via the muscle-specific pathway ([8] and results not shown).

Two considerations suggest that ASF/SF2 or a related SR protein could, in principle, control alternative splicing of

SERCA2 transcripts. First, muscle-specific SERCA2 splicing can be artificially induced by optimizing the 5' donor splice site, and ASF/SF2 can exert its effect by enhancing the binding of U1 small nuclear ribonuclear particles to non-consensus donor splice sites [29]. Secondly, SERCA2 contains, apart from the muscle-specific donor, a second donor splice site more proximal to the acceptor. This donor is only used in neuronal cells and it is a weak one [9]. The ASF/SF2 concentration can affect the choice of donor splice site when more than one splice donor competes for a common acceptor [27,30]. The prerequisite that both donor splice sites should be weak is fulfilled for SERCA2.

However, our observations do not support a critical role for ASF/SF2 or other SR proteins in SERCA2 processing. First, there is no noticeable increase in the expression of ASF/SF2 at the protein level during myogenic differentiation. We also found no difference in the concentration of two other 35 kDa SR proteins (9G8 and SC-35), while other SR proteins could not be detected. Secondly, overexpression of ASF/SF2 could not induce muscle-specific splicing in HeLa cells. It also did not up-regulate splicing when low levels of class-1 mRNA were already present in undifferentiated cells, neither did it induce neuronal splicing. A similar lack of effect of ASF/SF2 was reported for the muscle-specific alternative splice process in β -tropomyosin. Exon skipping could be influenced by ASF/SF2 overexpression, but the muscle-specific splice pattern with exon 7 present in the mature messenger could not be obtained [15]. Collectively, our data strongly indicate that SERCA2 processing is regulated by specific factors in a way that has not yet been clarified.

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