Epstein-Barr Virus SM Protein Functions as an Alternative Splicing Factor

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Alternative splicing of RNA increases the coding potential of the genome and allows for additional regulatory control over gene expression. The full extent of alternative splicing remains to be defined but is likely to significantly expand the size of the human transcriptome. There are several examples of mammalian viruses regulating viral splicing or inhibiting cellular splicing in order to facilitate viral replication. Here, we describe a viral protein that induces alternative splicing of a cellular RNA transcript. Epstein-Barr virus (EBV) SM protein is a viral protein essential for replication that enhances EBV gene expression by enhancing RNA stability and export. SM also increases cellular STAT1 expression, a central mediator of interferon signal transduction, but disproportionately increases the abundance of the STAT1β splicing isoform, which can act as a dominant-negative suppressor of STAT1α. SM induces splicing of STAT1 at a novel 5′ splice site, resulting in a STAT1 mRNA incapable of producing STAT1α. SM-induced alternative splicing is dependent on the presence of an RNA sequence to which SM binds directly and which can confer SM-dependent splicing on heterologous RNA. The cellular splicing factor ASF/SF2 also binds to this region and inhibits SM-RNA binding and SM-induced alternative splicing. These results suggest that viruses may regulate cellular gene expression at the level of alternative mRNA splicing in order to facilitate virus replication or persistence in vivo.

The Epstein-Barr virus (EBV) SM protein is expressed early in the lytic phase of virus replication and regulates EBV and cellular gene expression posttranscriptionally (for a review, see reference 36). SM is essential for efficient expression of EBV genes during early and late stages of lytic replication, EBV DNA replication, and virion production (3, 15, 16). SM protein binds to RNA and enhances export and stability of intronless EBV mRNAs (6, 12, 18, 30, 32, 35). SM interacts with cellular export proteins and is thought to act as a bridge between target mRNA and the cellular export machinery (4, 12, 19). However, no specific RNA sequence has been established as a unique target for SM binding.

The effects of SM on host cellular gene expression during lytic EBV replication remain to be fully characterized. When inductively expressed in EBV-negative cells, SM has a broadly inhibitory effect on cellular mRNA accumulation (31). Nevertheless, SM causes several cellular transcripts to accumulate at higher levels (31). These transcripts include STAT1 and several interferon (IFN)-stimulated genes. The STAT1 protein is an integral mediator of both type I (IFN-α/β) and type II (IFN-γ) IFN signal transduction pathways (for a review, see reference 10). STAT1 homodimers, when activated by IFN-γ, bind GAS sequences upstream of IFN-γ-responsive genes and stimulate transcription. Activated STAT1 also forms a trimeric complex with STAT2 and IRF-9 (IFN regulatory factor 9) and binds and activates transcription downstream of IFN-stimulated response element sequences, thereby mediating type I IFN signal transduction (13, 14, 33, 37). STAT1 is expressed as two isoforms, STAT1α and STAT1β (33). STAT1β mRNA is generated by cleavage and polyadenylation at an alternative site in the last intron of the STAT1 pre-mRNA, leading to production of a protein which lacks the trans-activating domain encoded in the last exon of the STAT1 gene (Fig. 1A). While STAT1β is still capable of transducing type I IFN signals, STAT1β homodimers, although capable of binding DNA, are not capable of activating GAS sequences and thus are inactive in IFN-γ signal transduction (25, 38). STAT1β may also heterodimerize with STAT1α, forming inactive dimers and thereby inhibiting STAT1α activity (2, 38). STAT1β therefore acts as a dominant-negative repressor of STAT1α signaling in the IFN-γ response (5, 29, 38). Consistent with a role for STAT1β as an antagonist of STAT1α, the ratio of STAT1α and β isoforms has been shown to affect cellular apoptosis and resistance to viral and mycobacterial infection (1, 2, 27). Interestingly, SM disproportionately increases the relative amounts of STAT1β mRNA, suggesting that SM also affects STAT1 mRNA processing (31). In order to investigate the possible role of SM protein in regulating STAT1 splicing, we studied the effects of SM on a STAT1 minigene composed of the relevant portions of the STAT1 gene.

MATERIALS AND METHODS

Plasmid constructions. To generate the STAT1 minigene construct, DV1, 1.088 kb of the STAT1 gene (accession number AY865620, nucleotides 40293 to 41381), which contains exon 23, exon 24, and the intron between exons 23 and 24, was amplified by high-fidelity PCR and cloned in mammalian expression vector pCDNA3 (Invitrogen) at the EcoRV site. To generate DV4 plasmid, 2.598 kb of DNA including the STAT1 3′ untranslated region (UTR) and polyadenylation signal (nucleotides 45381 to 47980) was amplified and cloned into DV1, replacing the plasmid bovine growth hormone polyadenylation signal. DV5 and DV6 plasmids, which were used to generate in vitro RNA transcripts of portions of the STAT1 gene, were generated by digestion of the 1.088-kb STAT1 fragment into 291 and 797 bp with DraI and cloned into the Bluescript M13+ (Stratagene)

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plasmid at the EcoRV site. Site-specific mutants and deletions were generated by overlapping PCR. The T7-tagged ASF/SF2 plasmid was kindly provided by Jeremy R Sanford. The pl-12 plasmid kindly provided by Mariano A. Garcia-Blanco has been previously described (23). The SRp20 plasmid was kindly provided by H. Lou (23). The SM expression plasmid was constructed by PCR amplification from B95-8 EBV DNA as previously described (32).

Deletions mutants of the SM response element were generated by PCR using AccuPrime Pfu DNA polymerase (Invitrogen) and cloned into the EcoRV site of pl-12 between exons U and D. PCR was performed using different 5' primers and a 3' primer that extended 25 bases downstream of the SM donor 5' splice site (SS). The sequence of each plasmid was verified by DNA sequencing. Primer sequences are available on request.

**Transfections and cell lines.** HeLa cells were maintained in Dulbecco’s modified Eagle medium containing 10% fetal calf serum. All transfections were performed with Lipofectamine Plus (Invitrogen) and cloned into the EcoRV site of pl-12 between exons U and D. PCR was performed using different 5' primers and a 3' primer that extended 25 bases downstream of the SM donor 5' splice site (SS). The sequence of each plasmid was verified by DNA sequencing. Primer sequences are available on request.

**RNA isolation and RT-PCR of minigenes.** Total RNA was isolated from cells with RNA-Bee (Teltest) and RNeasy columns (Qiagen) according to manufacturer’s protocols. Reverse transcription-PCR (RT-PCR) of the STAT1 minigene was performed using flanking region primers (Stat1HindIII, 5'-GCAAGCTTACACTATGTTCAATTTCTACAGACGCCC-3'; J1, 5'-GGGAGGATGCATAGAGAC-3'). Product sizes were confirmed by ethidium bromide staining. Quantification of RT-PCR products was performed using ImageQuant software (GE Healthcare), and linearity of measurement was confirmed with a standard curve generated with DNA fragments of known concentrations.

**RNA cross-linking assay.** Cos7 cells were transfected with vector plasmid, SM expression vector DNA, SRp20, or ASF/SF2 plasmids using Lipofectamine Plus (Invitrogen). At 48 h after transfection, cells were washed and harvested by scraping and centrifugation. Cell pellets were lysed by incubation at 4°C for 15 min in 100 μl of 20 mM HEPES (pH 7.9), 10 mM NaCl, 10% glycerol, 3 mM MgCl₂, 0.2 mM EDTA, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Sigma), with frequent mixing. The lysed cell suspension was centrifuged at 4°C for 5 min at 700 × g. Supernatant was transferred to a fresh tube, and a one-third volume of high-salt buffer (20 mM HEPES [pH 7.9], 400 mM KCl, 2% glycerol, 0.2 mM EDTA, 0.4 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and eukaryotic protease inhibitor cocktail (Sigma)) was added. Aliquots of extract were snap-frozen at −80°C. Radiolabeled DV1, DV5, and DV6 mRNAs were synthesized with [32P]UTP and T7 RNA polymerase. To allow RNA complex formation to occur, 2 × 10⁵ cpm of radiolabeled RNA was incubated for 30 min at 30°C with 8 μl of cell extract, 2 μl of 20 mM magnesium acetate, 2 μl of 10 mM ATP, 2 μl of 200 mM K glutamate, 2 μl of 50 mM creatine phosphate, 1 μl of RNA (1 μg/ml), and 1 μl of RNasin in a total volume of 20 μl. RNA-protein mixtures were then cross-linked by UV irradiation on ice with a Stratalinker (Stratagene) for a total of 0.6 J/cm². RNA was hydrolyzed by incubation with 100 μg of RNase A/ml for 1 h at 37°C. SM protein was immunoprecipitated from the incubation mixture and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

**RESULTS**

SM induces alternative splicing of a STAT1 mini gene. We have previously constructed an EBV-negative B lymphocyte cell line (SM18D) that inducibly expresses SM when treated with 4-hydroxy-tamoxifen (31). Microarray analysis of the transcriptional profile of this cell line demonstrated that cellular STAT1β levels are upregulated relative to STAT1α levels at

**FIG. 1.** Effect of SM on STAT1 pre-mRNA splicing. (A) Diagram of STAT1 minigene (DV1), which contains STAT1 exon 23, exon 24, and intervening sequence. Alternative processing events generate either the STAT1α isoform (exons 23 and 24); the SM-directed alternative α' isoform, in which the α 5' donor site is skipped and the α' donor 5' SS site is used; or the β isoform (cleavage and polyadenylation at canonical heptamer/pentamer in the last intron between exons 23 and 24) as indicated (CP). (B) SM induces alternative splicing of the STAT1 minigene. RNA from epithelial cells (HeLa) and B lymphocyte cells (BJAB) cotransfected with (+) and without (−) SM was analyzed by RT-PCR. Lane 1, 100-bp DNA molecular weight marker (M); lane 2, DV1 transfected with vector; lane 3, DV1 transfected with SM; lane 4, PCR without reverse transcriptase. The positions of PCR products corresponding to each splice product are indicated with arrows at right. The expected sizes of PCR products are 210 bp (α), 420 bp (α'), and 103 bp (β). PCR products were cloned and sequenced to confirm structure.
both the RNA and protein levels (31). In order to study how SM might affect processing of the STAT1 transcript, we generated a minigene template by cloning the relevant exons and intron of the STAT1 gene in the mammalian expression vector pCDNA3. This STAT1 minigene construct, named DV1, was used for in vivo splicing assays (Fig. 1A).

DV1 plasmid was transfected into epithelial (HeLa) and B lymphocyte (BJAB) cells with an SM expression vector or with vector alone. RNA was isolated 48 h after transfection and analyzed by RT-PCR using minigene flanking region primers. In the absence of SM, the major product was the constitutively spliced fragment expected from the splicing of exon 23 and exon 24 (Fig. 1B). Interestingly, in both cell types, SM led to production of a new spliced fragment in addition to the constitutively spliced fragment, which is larger than that expected from STAT1.

Cloning and sequencing of the alternatively spliced product, referred to as STAT1Δ/H9251/H11032, revealed that SM promoted use of a new 5′ SS 210 nucleotides downstream of the constitutive 5′ SS. The acceptor 3′ SS was the same as that used for the constitutive splicing that generates STAT1 (Fig. 2). The alternative 5′ SS has a canonical GT at the beginning of the intron.

STAT1Δ mRNA is generated by cleavage and polyadenylation at a canonical hexanucleotide (AAUAAA) in the intron between exons 23 and 24, as shown in Fig. 1A and Fig. 2. This premature cleavage and polyadenylation is predicted to result in termination of translation shortly after exon 23, resulting in the shorter STAT1Δ protein that lacks the transactivation domain of STAT1α. The alternative splicing induced by SM is predicted to generate an mRNA with a stop codon immediately following exon 23. Such an mRNA would either produce STAT1Δ protein after cleavage and polyadenylation distal to the final exon or be subject to nonsense-mediated decay (NMD) because of the distance between the stop codon and the α′ SS. Thus, the alternative splicing induced by SM may contribute to the increased STAT1Δ/STAT1α protein ratio previously described (31).

Polyadenylation and splicing are known to be linked processes (28). The polyadenylation signal present in the STAT1 minigene (DV4) was constructed in which 1.5 kb of 3′ UTR and 1 kb distal to the polyadenylation and cleavage signal of STAT1 were inserted 3′ to exons 23 and 24, replacing the vector-derived 3′ UTR and polyadenylation signal (upper panel). Epithelial cells (HeLa) and B lymphocyte cells (BJAB) were transfected with DV4 and either SM (+SM) or empty vector (−SM). RNA was analyzed by RT-PCR using STAT1-specific primers. Lane 1, 100-bp DNA molecular weight marker (M). The positions of PCR products corresponding to splice products are indicated. The expected sizes of PCR products are 210 bp (α′, constitutive splice product) and 420 bp (α′, SM-directed alternative splice product). PCR products were cloned, and splice junctions were confirmed by sequence analysis.

FIG. 2. Sequence of constitutive and SM-directed alternative STAT1Δ splice sites. Exons 23 and 24 are shown in uppercase letters and highlighted, and the intron is shown in lowercase letters. The italicized sequence is the additional sequence included in the upstream exon when SM induces skipping of the constitutive 5′ SS and splicing occurs at the alternative 5′ SS. The cleavage and polyadenylation signal AAUAAA and the site of polyadenylation resulting in STAT1Δ are highlighted and enclosed in boxes.

FIG. 3. SM effects on STAT1Δ minigene splicing are independent of polyadenylation signal and 3′ UTR sequences. A minigene plasmid (DV4) was constructed in which 1.5 kb of 3′ UTR and 1 kb distal to the polyadenylation and cleavage signal of STAT1 were inserted 3′ to exons 23 and 24, replacing the vector-derived 3′ UTR and polyadenylation signal (upper panel). Epithelial cells (HeLa) and B lymphocyte cells (BJAB) were transfected with DV4 and either SM (+SM) or empty vector (−SM). RNA was analyzed by RT-PCR using STAT1-specific primers. Lane 1, 100-bp DNA molecular weight marker (M). The positions of PCR products corresponding to splice products are indicated. The expected sizes of PCR products are 210 bp (α′, constitutive splice product) and 420 bp (α′, SM-directed alternative splice product). PCR products were cloned, and splice junctions were confirmed by sequence analysis.
SM induces alternative splicing of the cellular STAT1 gene. In order to determine whether SM induces alternative splicing of cellular STAT1 transcripts, we used SM18D cells that express SM protein from a 4-hydroxy-tamoxifen-inducible promoter (31). SM18D cells or SM-negative parent cells were grown in the presence of 4-hydroxy-tamoxifen, and RNA was isolated 48 h after SM induction and analyzed by RT-PCR with STAT1 primers. As shown in Fig. 4, SM induced alternative splicing of cellular STAT1 mRNA similar to its effect in the minigene system. The RT-PCR product corresponding to the alternatively spliced mRNA was sequenced and had the identical sequence to the unspliced pre-mRNA are indicated. The expected sizes of PCR products are 210 bp (α) and 410 bp (α'). PCR products were cloned and sequenced for verification of splice junctions. (C) Plasmid DV11 (alternative 5' SS mutated) was transfected and analyzed as in panel B above. The expected position of the α' SS and position of α SS are shown at left; molecular weight markers are at right. PCR products were sequenced for verification of splice junctions.

FIG. 5. Effect of SM on alternative SS usage from mutated STAT1 minigenes. (A) Diagrams of mutated constitutive (α) and alternate (α') 5' SSs of the STAT1 minigene. The constitutive 5' SS junction (GTGT) was replaced by GTAA (DV10), and the alternative 5' SS junction (AGGT) was replaced by ACAA (DV11). These substitutions are predicted to eliminate activity as a splice donor. (B) Plasmid DV10 (mutated constitutive 5' SS) was transfected with SM (+SM) or without SM (-SM), and RNA was analyzed by RT-PCR. The position of the α' splice product and the expected position of the α splice product are shown at right; molecular weight markers are at left. PCR products were sequenced for verification of splice junctions. (C) Plasmid DV11 (alternative 5' SS mutated) was transfected and analyzed as in panel B above. The expected position of the α' SS and position of α SS are shown at left; molecular weight markers are at right. PCR products were sequenced for verification of splice junctions.
side of each 5' SS were synthesized and substituted in the parent STAT1 minigene (Fig. 6A). The baseline pattern of splicing and the effect of SM on splicing of this construct were then analyzed. RT-PCR analysis of RNA from transfected cells showed that constitutive splicing was less efficient than with the parent STAT1 construct. However, SM still strongly activated the downstream SS (Fig. 6B). This suggests that the SM effect that directs splicing to the downstream SS requires elements in addition to those immediately surrounding the α' SS, i.e., that SM will not direct alternative splicing based on only the 50 nucleotides encompassing the α' SS.

**SM can activate alternative splicing in a heterologous system.** The finding that SM activated splicing of the α' 5' SS suggested that there were SM-responsive splicing enhancers in the STAT1 minigene. In order to further delineate the SM-responsive sequences, we asked whether the effect of SM on STAT1 minigene splicing was transferable to a heterologous system. We employed plasmid pl-12, containing two adenovirus-derived exons referred to as U and D, which has been previously characterized as a splicing substrate (8) (Fig. 7A). A STAT1 fragment containing potential SM response elements that included exon 23 of STAT1 and extended immediately 3' to the α' 5' SS was inserted into the intron of pl-12 between U and D. The α 5' SS in the STAT1 gene was inactivated in order to avoid competition between this 5' SS site and the U exon 5' SS. This construct (DV30) was transfected into HeLa cells with and without the SM expression plasmid, and splicing was analyzed by RT-PCR. As shown in Fig. 7B, lane 3, SM induced alternative splicing of DV30, resulting in the generation of a novel 512-nucleotide fragment. DNA sequencing revealed that the constitutive splice product obtained in the absence of SM resulted from ligation of the U and D exons, as expected. However, when SM was expressed, the α' 5' SS from STAT1 and the 3' SS of the D exon were utilized, extending the U exon to the α' 5' SS and resulting in the alternative splice product. These results demonstrate that sequences contained in exon 23 and the intron proximal to the α' 5' SS are sufficient for SM to direct alternative splicing and α' 5' SS utilization.

In order to further define the sequence requirements for SM-directed alternative STAT1 splicing, we constructed a series of minigenes consisting of successive 5' deletion mutants of the 319-bp STAT1 response element inserted in pl-12 (Fig. 7A). These constructs all terminate 25 bp 3' of the α' 5' SS but contain successive 5' deletions. The splicing pattern of these constructs in the presence or absence of SM was analyzed by RT-PCR using pl-12-specific primers, as shown in Fig. 7B. The percentage of alternative splicing of each minigene in the presence of SM is shown in Fig. 7C. The presence of 29 nucleotides of intronic sequence immediately 3' of exon 23 was essential for SM-directed utilization of the α' 5' SS. However, inclusion of additional exon 23 sequence up to 51 bp was necessary for maximal efficiency of SM-directed alternative splicing, suggesting that this 80-bp region of the STAT1 gene contains sequences important for SM activity.

**SM binds directly to STAT1 mRNA.** SM is known to interact with mRNA, although the sequence specificity, if any, of such binding remains to be established. In order to determine whether SM protein bound directly to STAT1 mRNA, we performed an in vitro RNA cross-linking assay by incubating cell extracts containing SM protein with radioactively labeled STAT1 transcript consisting of exons 23, intron, and exon 24. The mixture was UV irradiated, unbound RNA was hydrolyzed, and SM protein was immunoprecipitated. As shown in Fig. 8, SM protein was labeled by covalent linkage to radiolabeled uridine from STAT1 RNA, indicating that SM protein bound directly to STAT1 RNA, as expected. However, inclusion of additional exon 23 sequence up to 51 bp was necessary for maximal efficiency of SM-directed alternative splicing, suggesting that this 80-bp region of the STAT1 gene contains sequences important for SM activity.}

**SF2/ASF inhibits SM-induced alternative splicing and SM-RNA interaction.** Inspection of the sequence containing the SM response element using software to predict exonic splicing enhancers (ESEfinder [9]) revealed a cluster of binding sites for the human splicing factor ASF/SF2 (Fig. 9A). If SM binding to this region was required for directing alternative splicing to the α' 5' SS, we reasoned that SF2 might compete with SM for binding to the RNA and inhibit SM-directed alternative.
FIG. 7. SM activates alternative splicing in a heterologous system. (A) Schematic representation of various STAT1 constructs used in splicing experiments to map SM-responsive elements. Each fragment from the STAT1 gene (SMRE) was inserted between the U and D exons of pI-12 as shown. The expected spliced products in the absence of SM (constitutive) or presence of SM (SM-directed) are shown at right. If SM induces alternative splicing, a longer spliced product is expected due to inclusion of sequence up to the α′ SS in the upstream exon. The largest STAT1 fragment (DV30) includes the entire STAT1 exon 23 and extends 25 bp 3′ of the α′ SS. The solid boxes and lines represent exon and intron sequences, respectively. These constructs were transfected into HeLa cells with and without SM plasmid, and RNA was analyzed by RT-PCR. (B) Patterns of alternative splicing upon transfection with various STAT1 constructs (DVs 30, 34, 32, 14, 23, 22, and 16) with control plasmid (lanes 2, 4, 6, 8, 10, 12, and 14) or SM-expressing plasmid (lanes 3, 5, 7, 9, 11, 13, and 15). Asterisks denote the positions or expected positions of the SM-directed alternative splice products. The arrow marks the splice products generated by constitutive splicing of the U and D exons. The size of each expected product differs due to the variations in the extent of each deletion. Minor products seen above the spliced product represent unspliced pre-mRNA. Lane M, molecular weight ladder. (C) Quantitation of the extent of SM-induced alternative splicing in different constructs as shown in panel B. The amount of DNA in each species was quantitated by measurement of ethidium bromide-stained gels using ImageQuant software and standard curves using known amounts of double-stranded DNA fragments.
splicing. In order to directly test whether SF2 could inhibit SM binding to the STAT1 RNA, cross-linking assays were performed. Radioactive transcript of the RNA including exon 23 and extending to the α’ SS was cross-linked with lysate from cells transfected with SM alone or with SM and SF2. A parallel experiment was performed with SM and SRp20, another cellular SR (serine-arginine-rich) protein, as a control. As shown in Fig. 9B, SF2 inhibited SM binding to the STAT1 RNA, suggesting that SF2 binding sites overlap with the SM binding sites on the STAT1 RNA. This experiment was repeated, and lysates from each transfection were also immunoblotted in parallel to confirm that comparable amounts of SM were expressed when transfected with each splicing factor. We then tested the effect of SF2 overexpression on SM-mediated alternative splicing of the STAT1 minigene. HeLa cells were transfected with the STAT1 minigene in combination with either SM alone or with SM and SF2. A parallel experiment was performed with SM and SRp20, another cellular SR (serine-arginine-rich) protein, as a control. As shown in Fig. 9B, SF2 inhibited SM binding to the STAT1 RNA, suggesting that SF2 binding sites overlap with the SM binding sites on the STAT1 RNA. This experiment was repeated, and lysates from each transfection were also immunoblotted in parallel to confirm that comparable amounts of SM were expressed when transfected with each splicing factor. We then tested the effect of SF2 overexpression on SM-mediated alternative splicing of the STAT1 minigene. HeLa cells were transfected with the STAT1 minigene in combination with either SM alone or with SM and various amounts of SF2. Expression of SF2 inhibited SM-induced alternative splicing in a dose-dependent manner (Fig. 9C). These results suggested that SF2 inhibited SM-mediated alternative SS selection by competing for RNA binding and that binding of SM to this region is likely to be functionally important in alternative splicing. SRp20 cotransfection did not inhibit SM-mediated alternative splicing in similar experiments (data not shown).

**SM antagonizes IFN effects on STAT1 expression.** The SM-induced alternative STAT1 mRNA is predicted to either produce STAT1β protein or undergo NMD, due to the presence of a stop codon in the α’ mRNA immediately after exon 23. If SM leads to an increase in STAT1β and STAT1α’ mRNAs, the increase in the STAT1β/STAT1α protein ratio could act to modulate the effects of IFN signaling. In order to inquire whether SM affects the ratio of STAT1 protein isoforms produced in response to IFN treatment, we exposed SM18D cells to IFN-β, which induces STAT1 expression. As shown in Fig. 10, IFN treatment of SM18D cells led to increased expression of STAT1 protein, particularly of STAT1α, as expected. However, when SM was expressed, although total STAT1 levels were increased, STAT1β was produced in amounts equal to STAT1α. Importantly, when cells were treated with IFN in the presence of SM, SM maintained increased levels of STAT1β protein. The alteration in STAT1 isoform ratios suggests that SM may play a role in modulating the innate immune response by lowering the STAT1 α/β ratio of STAT1 produced during exposure of infected cells to IFN in vivo.

**DISCUSSION**

Viruses have evolved a number of mechanisms to modulate cellular splicing. Several mammalian DNA viruses inhibit cellular RNA splicing by inducing dephosphorylation of SR splicing cofactors, which also leads to changes in viral splicing.
The production of two isoforms of STAT1 protein by alternative cleavage and polyadenylation has been well described, and several lines of evidence indicate that the ratio of STAT1β to STAT1α modulates the cellular response to viral and mycobacterial infection, IFN exposure, and apoptotic stress (1, 2, 27, 38). We have previously shown that EBV SM leads to increased total STAT1 levels, resulting in increased expression of several genes stimulated by type I IFNs (IFN-α and IFN-β) (31). However, SM also increases the relative amount of the β form of the RNA, increasing the STAT1β/STAT1α RNA ratio (31), which has been shown to inhibit the ability of the cell to respond to IFN-γ (1, 5, 38). Demonstration of the specific effects of SM on the cellular IFN response will therefore require a comparative analysis of genes that are primarily responsive to IFN-γ but not to IFN-α/β, as there is considerable cross talk between type I and type II IFN signaling (11). Thus, most IFN-γ-responsive genes (ISGs) are also upregulated by increases in STAT1β via the STAT1/STAT2/IRF-9 complex in which STAT1β is active. Thus, the predicted effect of SM (which increases both STAT1α and β levels) on ISGs is that there will be a change in the relative levels of ISGs depending on how responsive they are to STAT1β, rather than an overall decrease in IFN-γ-responsive gene expression. The alternative SS induced by SM is thus reminiscent of recently described “poison cassette” exons present in SR protein genes themselves, which, when included as a result of alternative splicing, result in mRNAs that undergo NMD (22, 26). The extent to which SM-induced, alternatively spliced STAT1 mRNAs undergo NMD as opposed to translation remains to be determined.

The ability of a viral protein to influence cellular splicing and interact with cellular splicing factors by competing for binding to RNA or spliceosome components increases the potential for combinatorial diversity among splicing factors that are thought to regulate alternative splicing of greater than 70% of human genes (20). The presence of an alternative SS which is used only in the presence of a viral protein raises the possibility of yet another level of posttranscriptional regulation of cellular gene expression by infecting viruses and increases the potential complexity of a “splicing code.”

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