Research Article

Combined Use of MS2 and PP7 Coat Fusions Shows that TIA-1 Dominates hnRNP A1 for K-SAM Exon Splicing Control

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Splicing of the FGFR2 K-SAM exon is repressed by hnRNP A1 bound to the exon and activated by TIA-1 bound to the downstream intron. Both proteins are expressed similarly by cells whether they splice the exon or not, so it is important to know which one is dominant. To answer this question, we used bacteriophage PP7 and bacteriophage MS2 coat fusions to tether hnRNP A1 and TIA-1 to distinct sites on the same pre-mRNA molecule. hnRNP A1 fused to one coat protein was tethered to a K-SAM exon containing the corresponding coat protein’s binding site. TIA-1 fused to the other coat protein was tethered to the downstream intron containing that coat protein’s binding site. This led to efficient K-SAM exon splicing. Our results show that TIA-1 is dominant for K-SAM exon splicing control and validate the combined use of PP7 and MS2 coat proteins for studying posttranscriptional events.

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1. Introduction

Alternative splicing control is vital for correct gene expression, and it is important to understand how it works. We have been studying splicing of the mutually exclusive human FGFR2 alternative exons K-SAM and BEK [1–4]. The K-SAM exon is spliced in epithelial cells, while the BEK exon is spliced in mesenchymal cells. Deleting the BEK exon does not lead to efficient K-SAM exon splicing in mesenchymal cells, and deleting the K-SAM exon does not lead to efficient BEK exon splicing in epithelial cells [1]. Both exons are thus subject to at least partially independent splicing control. We have investigated the independent splicing control of the K-SAM exon and identified a major activator (TIA-1) and a major repressor (hnRNP A1) of this exon’s splicing. TIA-1 bound to an intron element (IAS1) immediately downstream from the K-SAM exon’s 5’ splice site activates splicing of the exon [5]. Two further downstream intron splicing enhancers (ISEs) IAS2 and IAS3 are also implicated in K-SAM exon splicing activation [4], hnRNP A1 bound to an exon splicing silencer (ESS) represses K-SAM exon splicing [6].

TIA-1 and hnRNP A1 are expressed by both epithelial and mesenchymal cells, so a number of different models can be formulated for K-SAM exon splicing control. For example, one model postulates that both proteins bind in mesenchymal cells, and that hnRNP A1 is dominant: K-SAM exon splicing is repressed. In this model, hnRNP A1 does not bind in epithelial cells, so the K-SAM exon is spliced. In another possible model both TIA-1 and hnRNP A1 bind to their sites in epithelial cells, but TIA-1 dominates and so K-SAM exon splicing is activated. In this second model, TIA-1 does not bind in mesenchymal cells, but hnRNP A1 does, so K-SAM exon splicing is repressed. To distinguish between these types of model we need to know if one of the two proteins is dominant, and if so, which one. Simple hnRNP A1 or TIA-1 overexpression studies cannot answer this question satisfactorily. Any effect observed could be indirect, involving binding of other proteins whose synthesis is induced by the overexpression protocol. This difficulty can however be overcome by tethering both hnRNP A1 to the exon and TIA-1 to the intron using heterologous RNA-binding domains.

Previously we used bacteriophage MS2 coat protein fusions to tether either hnRNP A1 [6] or TIA-1 [7] to a pre-mRNA molecule containing an RNA hairpin that binds MS2 coat. Tethering both proteins to the same pre-mRNA molecule implies using two fusion partners of different RNA
binding specificities. Coat protein from the *Pseudomonas aeruginosa* RNA bacteriophage PP7 binds to an RNA hairpin that differs from the MS2 hairpin in the position of a bulged adenosine and in the sequence of the loop [8]. As a result, each coat protein binds well to its own hairpin but shows very little affinity for the hairpin recognized by the other coat protein [9]. We investigate here the use of PP7 coat fusions for tethering proteins to RNA in transfected cells. We show that PP7 coat fusions function in the same way as the corresponding MS2 coat fusions, and that MS2 and PP7 coat fusions discriminate in favor of their cognate binding sites. This allowed us to use a combination of MS2 and PP7 coat fusions to put TIA-1 and hnRNP A1 in competition for control of K-SAM exon splicing, and to show that the activity of TIA-1 is dominant.

2. Material and Methods

2.1. Minigenes. RK97 has been described previously [5]. Minigenes S3 and S4 were made by replacing a PstI-XbaI fragment of RK97 containing IAS2 (which has no detectable activity in 293 cells) and some flanking sequences (nucleotides 80–214 of the intron downstream from the K-SAM exon) with annealed oligonucleotides coding, respectively, for single PP7 or MS2 RNA hairpins (sequences shown in Figure 1(b)). Minigenes S1 and S2 were made from a version of RK12 [2] deleted for the BEK exon, by replacing an EcoRI-EcoRV fragment from its CAT exon by annealed oligonucleotides coding for PP7 or MS2 RNA hairpins, respectively. The creation of any in frame termination codon was avoided. Minigenes S5 and S6 were made from S1 and S2, respectively, by replacing a PstI-XbaI fragment containing IAS2 (see above) with annealed oligonucleotides coding for MS2 or PP7 RNA hairpins, respectively.

2.2. Fusion Protein Vectors. MS2 coat sequences from pCI-MS2 [6] and PP7ΔFG coat sequences from a plasmid provided by Robert Singer, New York [10], were used to make sequences coding for MS2 coat dimers or PP7ΔFG dimers using standard techniques. Dimer coding sequences were introduced into a vector based on a version of pCI-neo (Promega) deleted for the neomycin resistance gene [6], and so were placed under control of a cytomegalovirus immediate-early promoter and the SV40 late polyadenylation signal. Dimer coding sequences are downstream from a vector sequence encoding a FLAG epitope tag separated from an SV40 nuclear localization signal by the in frame polylinker ATGCATAAGGGATATCCCTCGAGTCTAGA containing sites for NsiI, MluI, EcoRV, Xhol, and XbaI. TIA-1 and hnRNP A1 sequences from plasmids described in [5, 6], respectively, were used to make vectors expressing fusion proteins with coat dimers. During construction of these vectors, the SV40 NLS was deleted, as both TIA-1 and hnRNP A1 have their own NLS sequences. Constructs were verified by sequencing.

2.3. Transfections and RT-PCR. 5 × 10^5 293-EBNA cells (Invitrogen) were transfected by the calcium phosphate technique as described previously [11] with a total of 5 μg plasmid DNA: 0.5 μg minigene, with 0.02 μg TIA-1-coat fusion vector and/or 1 μg hnRNP A1-coat fusion vector added where appropriate, and pEGFP-1 (Clontech) used to bring the total up to 5 μg DNA. These conditions were chosen after different expression vector/minigene ratios were tested in preliminary experiments. They result in marked changes in splicing when cotransfecting minigenes with binding sites for a given fusion protein’s coat moiety and that protein’s expression vector, but no significant changes in splicing when the minigene used does not contain such a binding site. The need for high amounts of the hnRNP A1 fusion protein expression vector is most probably due to hnRNP A1 being one of the major cellular RNA binding proteins. Thus only a minor fraction of the hnRNP A1 fusion proteins synthesized will be available for binding to minigene RNA using the coat moiety, most molecules will use their functional hnRNP A1 moieties to bind to cellular RNAs.
RNA was harvested and analysed by RT-PCR using primers P1 and P2 [11]. 26–28 cycles of amplification were used to remain in the exponential amplification range. Products were migrated on 2% agarose gels, transferred to Nylon filters (Hybond N+, Amersham Biosciences), and hybridized with a $^{32}$P-labelled linked C1 and C2 probe. Filters were subjected to autoradiography or used for PhosphorImager analysis (PhosphorImager 445SI, Amersham Biosciences) with quantification using Image-Quant software.

3. Results and Discussion

3.1. hnRNP A1 and TIA-1 Fused to Coat Dimers. We wished to tether just one copy of TIA-1 or hnRNP A1 to a pre-mRNA. However, as MS2 and PP7 coat proteins bind to cognate hairpins as dimers, two copies of a protein fused to a coat monomer will be tethered per RNA hairpin. Tethering just one copy requires fusing the protein to a coat dimer. Such dimers can be obtained by joining two coat proteins together with a short linker [12]. Interactions between coat dimers can be avoided using MS2 coat with V75E A81G mutations [13], and PP7 coat deleted for amino acids 67–75 (PP7ΔFG) [10]. We constructed expression vectors for the production of PP7ΔFG or MS2 coat V75E A81G dimers with an N-terminal FLAG epitope tag and an SV40 nuclear localization signal (PP and MM, resp., Figure 1(a)). The vectors contain a polylinker for the insertion of additional coding sequences between the FLAG tag and the nuclear localization signal (PP and MM, resp., Figure 1(a)). The vectors contain a polylinker for the insertion of additional coding sequences between the FLAG tag and the nuclear localization signal and were used to make additional vectors coding for hnRNP A1 fusions (A1-PP and A1-MM) and TIA-1 fusions (TIA-PP and TIA-MM). As hnRNP A1 and TIA-1 have their own nuclear localization signals, the sequences coding for the SV40 nuclear localization signal were removed from the hnRNP A1 and TIA-1 fusion vectors. The expected RNA binding specificities of these proteins is shown in Figure 1(b).

Western blotting of protein extracts from transfected 293 cells with the FLAG M2 antibody showed that the proteins were synthesized correctly (data not shown).

3.2. Tethered hnRNP A1-PP7 Coat Fusion Represses Splicing Specifically. Our first aim was to test if hnRNP A1 and TIA-1 fusions with PP7 coat reproduce the behaviour we have already described for the corresponding MS2 coat fusions [6, 7]. For this, we constructed various FGFR2 minigenes containing different K-SAM-based exons together with flanking C1 and C2 exons and the corresponding introns (Figure 2). We have shown previously that replacing most of the K-SAM internal exon sequences (including the ESS) by bacterial CAT-encoding sequences (mutated to include an EcoRV and a SalI site) leads to efficient K-SAM exon splicing in mesenchymal cells (minigene RK12 [2]). However, replacing an RK12 EcoRV-SalI CAT fragment by sequences containing the K-SAM exon’s ESS yields an exon whose splicing faithfully reproduces that of the K-SAM exon [3, 4]. The RK12 system is thus very convenient for testing a variety of sequences for their ability to repress K-SAM exon splicing. Minigenes S1 and S2 were made by replacing the RK12 CAT exon’s EcoRV-SalI fragment by the PP7 or the MS2 hairpin, respectively, to permit tethering of A1-PP or A1-MM to the exon. The minigenes are also deleted for the competing BEK exon, so a simple RT-PCR protocol using minigene-specific primers P1 and P2 can be used to measure inclusion or skipping of their K-SAM-derived “CAT exon.”

293 cells (that do not usually splice the K-SAM exon) were transfected with S1 or S2, and RNA was harvested and analyzed by RT-PCR using primers P1 and P2. For these minigenes, the CAT exon is spliced efficiently as expected for an exon with no ESS (Figure 3(a), lanes 1 and 5). To determine the effect of tethering A1-PP or A1-MM, S1 and S2 were cotransfected with expression vectors for these...
fusion proteins. Splicing of the S1 CAT-PP7 exon is repressed by cotransfecting the A1-PP vector (compare lane 3 to lanes 1 and 2) to the same extent as splicing of the S2 CAT-MS2 exon is repressed by cotransfecting the A1-MM vector (compare lane 7 to lanes 5 and 6). Most importantly, splicing of the S1 CAT-PP7 exon is not repressed by cotransfecting the A1-MM vector (compare lane 4 to lanes 1 and 2), nor is the splicing of the S2 CAT-MS2 exon repressed by cotransfecting the A1-PP vector (compare lane 8 to lanes 5 and 6). These results demonstrate that repression cannot be attributed to some indirect effect of overexpression of the hnRNP A1 moiety, but that it requires tethering of the hnRNP A1 moiety to the exon. Furthermore, the results show that each fusion discriminates well in favour of its cognate RNA hairpin, and that the hnRNP A1-PP7 coat fusion reproduces the behavior of the corresponding MS2 coat fusion.

3.3. Tethered TIA-1-PP7 Coat Fusion Activates Splicing. To test if tethered TIA-PP can activate splicing as well as TIA-MM, we used minigenes S3 and S4 (Figure 2). These minigenes contain IAS1 and carry the K-SAM exon linked to sequences coding for a PP7 hairpin (S3) or an MS2 hairpin (S4) in the downstream intron. What is the rationale for using these minigenes rather than minigenes in which IAS1 is replaced by one of the hairpins? In fact, these latter minigenes are not useful here, as TIA-1-coat fusion proteins do not activate splicing when recruited to a hairpin replacing IAS1 (our unpublished results). For splicing activation, TIA-1 bound to the IAS1 element immediately downstream from the 5′ splice site needs to contact U1 snRNP bound to the 5′ splice site [14]. Precise positioning of TIA-1’s RRMs relative to the 5′ splice site is necessary for this contact. This positioning can only be obtained by TIA-1 interacting with IAS1, so TIA-1 tethered to a hairpin replacing IAS1 will not activate splicing. However, we have shown elsewhere that tethering a TIA-1-MS2 coat fusion to an intron site downstream from IAS1 activates K-SAM exon splicing in 293 cells [7]. Activation is strictly IAS1-dependent, implying that tethering increases the local concentration of TIA-1 around IAS1 and allows tethered TIA-1 to bind to IAS1, and thus interact with U1 snRNP to activate splicing.

293 cells were cotransfected with either S3 or S4 and RNA harvested and analyzed. For these minigenes, the K-SAM exon is not spliced efficiently (Figure 3(b), lanes 1 and 6) as it contains a functional ESS. To determine the effect of tethering TIA-PP or TIA-MM, S3 and S4 were cotransfected with expression vectors for these fusion proteins. Splicing of the K-SAM exon linked to an intronic PP7 hairpin (S3) is activated by cotransfecting the TIA-PP vector (compare lane 3 to lanes 1 and 2) and splicing of the K-SAM exon linked to an intronic MS2 hairpin (S4) is activated by cotransfecting the TIA-MM vector (compare lane 8 to lanes 6 and 7).
RNA was analyzed as described in the legend of Figure 3. PP7 coat dimers (PP), MS2 coat dimers (MM), TIA-1 fused to PP7 coat dimers (TIA-PP), or TIA-1 fused to MS2 coat dimers (TIA-MM).

Figure 4: TIA-1 dominance over hnRNP A1. 293 cells were cotransfected with minigenes S1, S2, S5, or S6 as marked and vectors coding for PP7 coat dimers (PP), MS2 coat dimers (MM), TIA-1 fused to PP7 coat dimers (TIA-PP), or TIA-1 fused to MS2 coat dimers (TIA-MM). RNA was analyzed as described in the legend of Figure 3.

At the amount of expression vector used in these experiments, TIA-MM and TIA-PP only marginally increase splicing of the K-SAM exon linked to a PP7 (S3) and an MS2 hairpin (S4), respectively, where tethering is not possible (Figure 3(b), lanes 5 and 10). We do not believe that this reflects low-level binding of fusions to their noncognate hairpins, but rather low-level binding of their TIA-1 moieties to IAS1 resulting from the increase in TIA-1 concentration provoked by transfection of the TIA-1 fusions. Thus we have shown elsewhere that increasing the TIA-1 concentration by provoking by transfection of the TIA-1 fusions. Thus we have shown elsewhere that increasing the TIA-1 concentration by transfection induces splicing of K-SAM exons linked to IAS1 [5]; the S3 and S4 K-SAM exons are linked to IAS1 (Figure 2). Furthermore, our results with hnRNPA1 fusions (see above) provide no evidence in favour of binding to noncognate hairpins.

3.4. Tethered TIA-1 Dominates Tethered hnRNP A1. Tethered hnRNPA1 represses K-SAM exon splicing, while tethered TIA-1 activates K-SAM exon splicing. What happens if hnRNPA1 and TIA-1 are tethered simultaneously? Minigenes S5 and S6 were designed for tethering an hnRNPA1 fusion to the CAT exon, and a TIA-1 fusion to the downstream intron sequences (Figure 2). These minigenes do not contain an ESS in the CAT exon, so their CAT exons are spliced efficiently in 293 cells (Figure 4, lanes 1 and 6). As expected, tethering A1-PP to the S5 CAT-PP7 exon reduces its splicing significantly (compare lane 3 to lanes 1 and 2).

Repression by tethered A1-PP is almost completely abrogated by the additional cotransfection of TIA-MM (compare lane 4 to lanes 2 and 3). This abrogation requires tethering of TIA-MM to the intron, as cotransfection of TIA-MM does not abrogate splicing repression by tethered A1-PP in experiments with the S1 minigene, which contains an A1-MM but not a TIA-PP binding site (lane 5). We can therefore rule out any explanation based on TIA-MM expression reducing the amount of A1-PP to levels too low for splicing repression.

Can these results be reproduced if the PP7 and MS2 hairpins are swopped (minigenes S6, Figure 4)? The effects of tethering unfused MM or PP to CAT exons is in general minor (minigenes S1, S2 and S5, Figures 3(a) and 4). Tethering MM to the S6 CAT-MS2 exon has a larger effect on splicing (reduction from 98% to 58%, compare lane 7 to lane 6). We can provide no explanation for this. However, a much greater reduction in splicing is obtained if A1-MM is tethered (from 98% to 13%, compare lane 8 to lanes 6 and 7). It is important to make the distinction between the part of this reduction (from 98% to 58%) that can be attributed to the tethering of A1-MM’s MM moiety, and the part (from 58% to 13%) that can be attributed to the tethering of the A1 moiety. The reduction in splicing attributable to the tethered A1 moiety of A1-MM is almost completely eliminated if TIA-PP is cotransfected: the level of splicing is restored from 13% to 49%. This is close to that observed (58%) when tethering MM alone (compare lane 9 to lane 7). Note that we cannot expect the level of splicing to be restored to 98% here. There is no reason for tethered TIA-1 to be able to eliminate the repression exerted by A1-MM’s MM moiety.

The abrogation of splicing repression by the A1 moiety of tethered A1-MM requires tethering of TIA-PP to the intron, as cotransfection of TIA-PP does not abrogate splicing repression in experiments with the S2 minigene, which contains an A1-MM but not a TIA-PP binding site (lane 10). We can therefore rule out any explanation based on TIA-MM expression reducing the amount of A1-PP to levels too low for splicing repression.

4. Conclusions

We conclude that when TIA-1 and hnRNPA1 can both bind and compete for control of K-SAM exon splicing, the activity of TIA-1 is dominant. Normally, TIA-1 bound just downstream from an exon’s suboptimal 5’ splice site helps recruit U1 snRNP to the splice site [14]. If TIA-1 is dominant, tethered hnRNPA1 should not repress splicing of an exon whose 5’ splice site has been rendered optimal by mutation. This is indeed the case: A1-PP no longer represses splicing of the S1 minigene’s CAT-PP7 exon if the exon’s 5’ splice site has been optimised (data not shown). We propose the following model for K-SAM exon splicing. In epithelial cells, TIA-1 binds to IAS1 and prevents hnRNPA1 from repressing K-SAM exon splicing, either by overcoming the effect of ESS-bound hnRNPA1, or by preventing hnRNPA1 binding to the K-SAM ESS. The K-SAM exon is spliced. In mesenchymal
cells, hnRNP A1 binds to the ESS, but TIA-1 does not bind to IAS1. K-SAM exon splicing is repressed. Why does TIA-1 bind to IAS1 in epithelial cells but not in mesenchymal cells? Splicing of the K-SAM exon is activated in epithelial cells by two intron splicing enhancers (IAS 2 and 3) in addition to IAS1 [4]. It is possible that these enhancers recruit proteins that work at least in part by tethering TIA-1 close to IAS1, and thus favour its binding to IAS1.

To conclude, we note that approaches involving tethering MS2 coat fusions or fusions to the bacteriophage lambda N protein to RNA molecules have benefited studies of posttranscriptional events including not only splicing but also polyadenylation, translation, RNA localization, and RNA stability [15–23]. These events often implicate several RNA binding proteins. The approach we have described here involving the combined use of PP7 and MS2 fusions should thus be of general interest, and if extended to include N protein fusions, should allow tethering of three different proteins to an RNA molecule.

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