

Polypyrimidine Tract-Binding Protein Down-Regulates Fibroblast Growth Factor Receptor 1 α -Exon Inclusion¹

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Abstract

Exclusion of the α -exon by alternative RNA splicing of the fibroblast growth factor receptor 1 (FGFR1) primary transcript leads to the production of FGFR1 β . Glial cell transformation is associated with a progressive increase in FGFR1 β expression that coincides with a dramatic increase in the expression of the splicing factor polypyrimidine tract-binding protein (PTB). Cell-specific overexpression of PTB increased α -exon skipping, and a reduction in PTB increased α -exon inclusion. Targeted disruption of PTB interaction with FGFR1 precursor RNA *in vivo* by an antisense oligonucleotide also increased α -exon inclusion. These results suggest that PTB plays a direct role in α -exon splicing.

Introduction

Aberrant activation of the FGF⁴ signaling pathway through overexpression of fibroblast growth factors and their cognate receptors is associated with several cancer phenotypes (reviewed in Ref. 1). In glioblastomas, overexpression of the FGFR1 receptor is also associated with a loss of RNA splicing regulation, whereby a single exon (α -exon) is predominantly skipped to produce the FGFR1 β isoform (2). The consequence of this change in splicing remains unclear. The FGFR1 β isoform has been shown to have increased affinity for FGF1 and FGF2, which possibly confers a growth advantage to tumor cells (3). Other studies suggest that the two receptor isoforms differ in their subcellular localization and that nuclear localization may play a major role in cell proliferation (4, 5). Unfortunately, conflicting data exist for glioblastoma cells (6). Production of the FGFR1 β isoform is dependent on two intronic sequences flanking the α -exon (7). The upstream sequence specifically binds the splicing inhibitory factor PTB, which is dramatically overexpressed in malignant glioblastomas (8). This implies a role for this protein in FGFR1 splicing. In the present study, we examined the effect of modifying PTB expression and blocking access to the upstream intronic element on the regulation of α -exon splicing.

Materials and Methods

Cell Culture. P19 cells (mouse embryonic carcinoma cells; ATCC, Manassas, VA), human normal astrocyte cells (Cambrex Biosciences, Baltimore, MD), and SNB-19 cells (human glioblastoma cells) were maintained according to supplier guidelines or previously described methods (8). Transfection experiments were performed in 6-well plates using GenePORTER 2 transfection

reagent (Gene Therapy Systems, Inc., San Diego, CA). In antisense experiments, SNB-19 cells were initially transfected with episomal vectors, followed by a 7–14-day treatment with 400 μ g/ml G418 before transfection of the RNA splicing reporter construct. The P19 cells were differentiated by plating at an initial density of 10⁵ cells/ml in bacteriological grade Petri dishes and treatment for 96 h with 0.5 μ M retinoic acid (Sigma, St. Louis, MO) with a single change of medium at 48 h. The cells were then washed three times with serum-free medium, and ~100 aggregates were transferred into 100-mm tissue culture dishes and grown in the absence of retinoic acid (9). RNA and protein analyses were performed 3 and 5 days after the transfer to the tissue culture plates, with the transfection of the splicing reporter construct occurring 48 h before sampling.

Western Blot Analysis. Western blot analysis was performed as described previously (8). The PTB antibody was a generous gift from Dr. Douglas Black (Howard Hughes Medical Institute at UCLA, Los Angeles, CA) (10). The α -actin antibody was purchased from Amersham Biosciences (Piscataway, NJ).

Plasmid Construction. FGFR1 minigene constructs pFGFR-17 and pFGFR-57 and the pHisG-PTB expression construct (a generous gift from Drs. Eric J. Wagner and Mariano Garcia-Blanco, Duke University Medical Center, Durham, NC) have been described previously (7, 11). The PTB antisense construct was created by insertion of a DNA fragment encoding nucleotides –5 to 986 relative to the translation start site into the episomal vector pCEP4 (Invitrogen Life Technologies, Inc., Carlsbad, CA).

RT-PCR, Oligonucleotide Treatment, and UV Cross-linking. RT-PCR analysis was performed as described previously with ³²P-end-labeled DS8 forward primer and hMT2/3 for 20 PCR cycles (8). *In vitro* and *in vivo* blocking experiments were performed using either the ISS-1-specific antisense (5'-CGACGAAGGAUUGAAACGGAGAAA-3') or random (5'-CCUCU-UACCUCAGUUACAAUUUUAU-3') 2'-O-methyl-modified phosphorothioate oligoribonucleotides (The Midland Certified Reagent Company, Midland, TX). For UV cross-linking experiments, the RNA oligomer was added to radiolabeled ISS-1 RNA on ice before the addition of SNB-19 nuclear extract. UV cross-linking and detection were performed as described previously (8). *In vivo* RNA oligomer treatment was performed on SNB-19 cells by transfection using Oligofectamine (Invitrogen Life Technologies, Inc. Carlsbad, CA). RNA was isolated 48 h after transfection, and endogenous FGFR1 α -exon splicing was examined with RT-PCR using primers FP172 (5'-GGAAGTGCTCCTCTTCTGG-3') and FP173 (5'-TTATGATGCTCCAGGTGGCA-3'), with 24 PCR cycles.

Results and Discussion

Although aberrant RNA splicing of FGFR1 correlates with increasing glial cell malignancy, the mechanism and significance of this observation remain unclear. We previously identified a 40-nucleotide intronic silencer sequence (ISS-1) that is required for glioblastoma cell-specific α -exon skipping and specifically binds to the splicing inhibitory protein PTB (Refs. 7, 8; Fig. 1A). We also found that the expression of PTB is dramatically higher in glioblastoma tissue than in normal adjacent tissue. The present study examined whether PTB plays a direct role in maintaining tumor-specific α -exon skipping. Western blot analysis revealed that the human astrocytoma cell line SNB-19 expressed a high level of PTB, comparable to that of human tumor samples (Fig. 1B and data not shown). Primary normal human astrocytes and the mouse embryonic carcinoma cell line P19 ex-

Received 5/16/03; revised 7/22/03; accepted 7/24/03.

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¹ This work was supported in part by NIH Grant CA67946 (to G. J. C.).

² I. G. B. was supported by Grant 003657-0147-1999 from the Texas Higher Education Coordinating Board.

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⁴ The abbreviations used are: FGF, fibroblast factor; FGFR, fibroblast growth factor receptor; PTB, polypyrimidine tract binding protein; ISS, intronic splicing silencer; RT-PCR, reverse transcription-PCR.

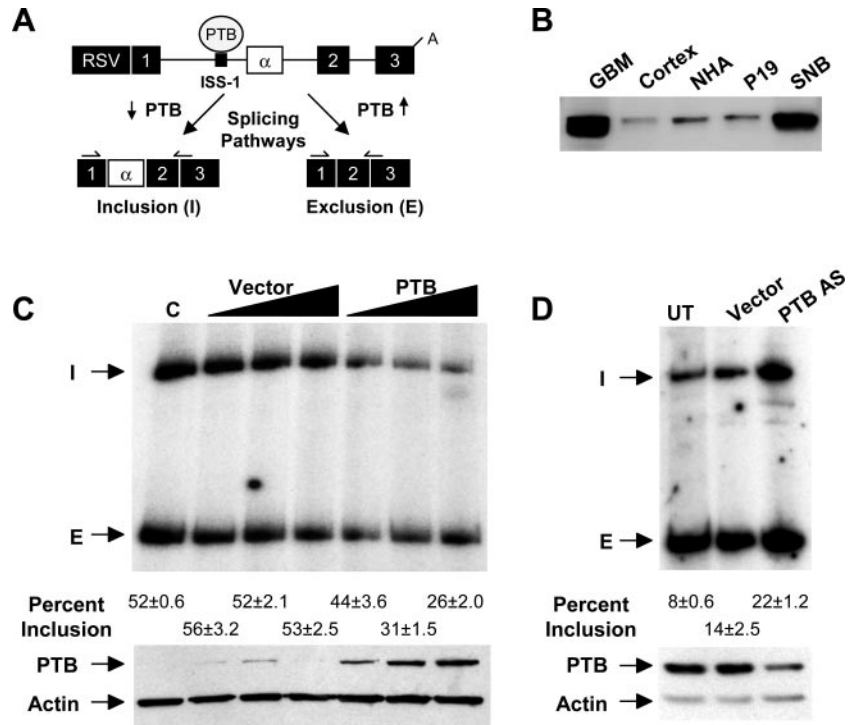


Fig. 1. PTB expression levels correlate with FGFR1 splicing. *A*, the FGFR1 minigene construct pFGFR-17, showing the proposed splicing model for PTB-mediated exclusion of the α -exon. *B*, Western blot using anti-PTB antibody. Each lane contained 10 μ g of total protein from a human glioblastoma tumor (GBM), adjacent normal brain (Cortex), normal human astrocytes (NHA), P19 cells, or SNB-19 cells (SNB). *C*, P19 cells were transfected with 0.5 μ g of pFGFR-17 alone (Lane C) or cotransfected with increasing amounts (0.5, 1, or 1.5 μ g) of pcDNA 3.1 (Vector) or PTB expression construct (PTB). RT-PCR (top) or Western blot analysis (bottom) was performed 48 h after transfection. Quantification of α -exon inclusion for three independent transfections (\pm SE) is shown below each lane. *D*, SNB-19 cells (UT), cells previously transfected with pCEP4 plasmid (Vector), or cells previously transfected with a vector expressing PTB antisense (PTB AS) were transfected with a pFGFR-17 splicing reporter construct (see "Materials and Methods"). RT-PCR (top) or Western blot analysis (bottom) was performed as described for panel C. *I*, inclusion of α exon; *E*, exclusion of α exon.

pressed low levels of PTB, similar to those observed in normal brain cortex (Fig. 1*B*). Because of transfection difficulties with the primary astrocyte cells, we chose the P19 cell line for further analysis.

To establish a link between the PTB level and FGFR1 splicing, we modified the PTB expression level in P19 and SNB-19 cells. P19 cells were cotransfected with an FGFR1 splicing reporter (pFGFR-17) and either a PTB expression plasmid (pHisG-PTB) or a vector control (pcDNA 3.1). RT-PCR and Western blot analysis revealed that increased expression of PTB directly correlated with reduced inclusion of the α -exon. In P19 cells transfected with pFGFR-17 alone, the average level of α -exon inclusion was 52% (Fig. 1*C*). Overexpression of PTB reduced α -exon inclusion to as low as 26%, whereas the vector control had no effect on splicing (Fig. 1*C*). The lack of a further reduction in exon inclusion might have resulted from a limiting amount of a corepressor. In SNB-19 cells, a reduction in PTB expression correlated with enhanced α -exon inclusion. To reduce endogenous PTB levels, we transfected SNB-19 cells with an episomal antisense vector (PTB AS) and subjected them to a 7–14-day selection in G418. Transfection of pFGFR-17 into selected cells gave a specific correlation of PTB reduction with an increase in α -exon inclusion, from 8% in control cells to 22% in PTB antisense cells (Fig. 1*D*).

To confirm that the PTB effects observed in P19 and SNB-19 cells were mediated by the ISS-1 regulatory element, we repeated the transfection experiments, using the splicing reporter construct pFGFR-54, which lacks the ISS-1 element. In transfected P19 cells, deletion of the ISS-1 led to a significant increase in the level of α -exon inclusion (compare Fig. 1*C* and Fig. 2*A*). Therefore, the ISS-1 element retained some inhibitory function in P19 cells, perhaps because of low levels of PTB or the involvement of additional negative regulatory factors. However, overexpression of PTB through cotransfection failed to reduce α -exon inclusion, supporting the hypothesis that the effects of PTB on splicing were primarily mediated through the ISS-1 element (Fig. 2*A*). Additional support for this was provided by experiments performed in SNB-19 cells, where PTB antisense expression had no effect on the splicing of transfected pFGFR-54 compared with control cells (Fig. 2*B*). Together, these results clearly

showed that PTB-mediated inhibition of α -exon inclusion depends on the presence of the ISS-1 element.

The enhancement of α -exon inclusion observed in P19 cells transfected with pFGFR-54 suggests that even small amounts of PTB inhibit exon recognition, that additional regulatory factors mediate the ISS-1-dependent response, or both. Previous studies have shown that the differentiation of P19 cells into a neural cell-enriched population further reduces PTB levels (10). Retinoic acid differentiation of P19 cells reduced PTB levels as detected by Western blot analysis, and this coincided with the appearance of neural cell types (Fig. 3). This PTB reduction produced a marked enhancement in the ratio of α -exon inclusion relative to skipping (Fig. 3*A*). Whether this change in splicing is entirely dependent on changes in the PTB level is unclear, because the levels of other splicing regulators have also been reported to change during P19 cell differentiation (12).

The transfection data presented here clearly support a role for PTB in FGFR1 α -exon exclusion. However, they do not show whether the interaction of PTB with the ISS-1 sequence of the endogenous FGFR1 mRNA precursor also enhances α -exon exclusion. To explore this

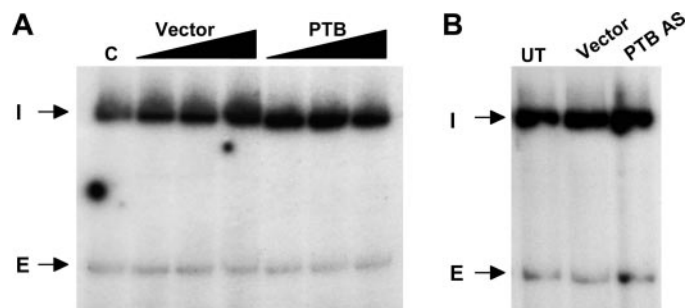


Fig. 2. Effect of ISS-1 deletion on PTB-mediated RNA splicing. *A*, P19 cells were transfected with pFGFR-54 alone (0.5 μ g; Lane C), or with increasing amounts (0.5, 1 or 1.5 μ g) of pcDNA 3.1 (Vector) or PTB expression construct (PTB). *B*, SNB-19 cells (UT), cells maintaining pCEP4 plasmid (Vector), or cells expressing PTB antisense (PTB AS) were transfected with pFGFR-54 splicing reporter construct. RT-PCR was performed as described in the legend for Fig. 1. *I*, inclusion of α exon; *E*, exclusion of α exon.

possibility, we adapted a targeted RNA antisense approach previously used to block splice site recognition (13). UV cross-linking experiments with SNB-19 nuclear extract showed that an ISS-1 antisense RNA oligonucleotide (ISS-1AS) blocked PTB interaction with the element in a concentration-dependent manner (Fig. 4A). A random oligonucleotide had no effect on PTB cross-linking. Transfection of the ISS-1AS oligonucleotide into SNB-19 cells caused a modest but reproducible increase in α -exon inclusion, as detected by RT-PCR (Fig. 4B). No change in endogenous *FGFR1* splicing was observed after transfection of a random RNA oligonucleotide (Fig. 4B). It is unclear whether the inability to induce a greater increase in exon inclusion results from reduced accessibility of the RNA oligonucleotide *in vivo*, from a greater role for other regulatory sequences such as ISS-2 (7), or from masking of the magnitude of the effect by the presence of steady-state spliced *FGFR1* mRNA.

Among the many regulators of alternative RNA splicing, PTB has emerged as key regulator of exon exclusion. It has been proposed that PTB-mediated exon exclusion works primarily by preventing spliceosome access (14). For some genes, such as *GABA_A γ 2*, the binding of PTB to sequences within the 3' splice site region effectively block U2AF recognition and ultimately spliceosome formation (15). However, for most genes, such as *FGFR1*, the PTB-binding sites are distal to the exon splice sites. For these genes, it has been postulated that the initial binding of PTB to regulatory sites serves as a nucleus for the multimerization of PTB and other regulatory proteins (14). The expansion of this PTB-containing protein complex then sequesters the specific exon from spliceosome recognition (14). This model is consistent with our findings. First, although the overexpression of PTB *in vivo* does lead to a reduction in α -exon inclusion, like other PTB-regulated genes it never approaches the reduction induced by the deletion of ISS-1. The same is true for *in vivo* reduction of PTB. For example, studies applying RNAi technology have shown that a reduction in endogenous PTB in rat prostate cancer cells significantly enhances inclusion of the IIIb exon of *FGFR2*, but this reduction was only about one-third as effective as the deletion of the *cis*-regulatory elements (16). Both observations clearly support a role for factors in addition to PTB playing a critical role in the repression of exon recognition.

In previous studies, we identified a direct correlation between the overexpression of PTB in glioblastomas and enhanced exclusion of the *FGFR1* α -exon during splicing (8). This study provides evidence that PTB is capable of mediating α -exon recognition by interacting with the ISS-1 element and that blocking this interaction alters endogenous *FGFR1* gene splicing. Changes in the PTB level alone do

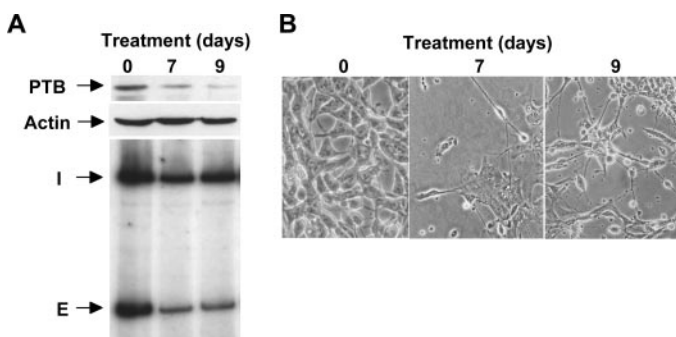


Fig. 3. Retinoic acid differentiation of P19 cells is associated with reduced PTB expression and altered *FGFR1* splicing. *A*, untreated P19 cells transfected with 1 μ g of pFGFR-17 served as a control (Lane 0). In treated cells, transfections were performed on days 5 and 7, with RT-PCR (bottom) or Western blot analysis (top) performed on days 7 and 9 after the initiation of retinoic acid treatment. *I*, inclusion of α exon; *E*, exclusion of α exon. *B*, morphology of P19 cells after retinoic acid treatment. Micrographs of untreated P19 cells (0) and cells on days 7 and 9 after the initiation of treatment ($\times 400$ magnification).

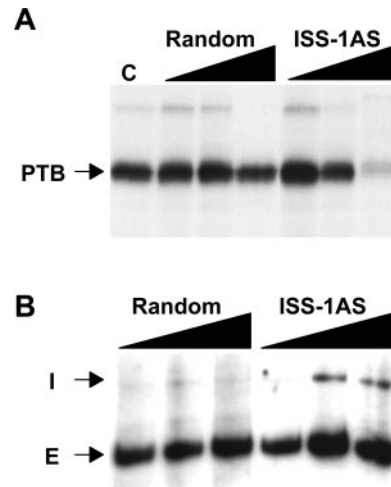


Fig. 4. Antisense RNA oligonucleotide targeting the ISS-1 element inhibits PTB binding and enhances endogenous α -exon inclusion. *A*, *in vitro* inhibition of PTB binding to the ISS-1 sequence. Random or ISS-1 antisense RNA (ISS-1AS) oligonucleotides were preincubated with a radiolabeled ISS-1 transcript at molar ratios of $\sim 1:1.5$, $2:1$, and $20:1$ unlabeled to labeled RNA. SNB-19 cell nuclear extracts were then added and incubated under *in vitro* splicing conditions for 10 min, followed by a UV cross-linking assay. No RNA oligonucleotide was added to the control reaction (Lane C). *B*, SNB-19 cells were transfected with 0.05, 0.1, and 0.2 μ M (final concentration) of random or ISS-1 antisense RNA oligonucleotide. RT-PCR examining α -exon splicing of endogenous *FGFR1* gene transcripts was performed 48 h after transfection as described in "Materials and Methods." *I*, inclusion of α exon; *E*, exclusion of α exon.

not appear to be responsible for the aberrant *FGFR1* splicing, suggesting that additional regulators of splicing must certainly be involved. However, it is interesting to note that SNB-19 cells transfected with PTB antisense vector show reduced colony formation in soft agar.⁵ Whether changes in *FGFR1* splicing play a role in this effect is not known. The ability to change splicing by antisense oligonucleotide treatment provides a method to specifically test the impact of a reduction in *FGFR1* β isoform on glioblastoma cell growth.

Acknowledgments

We appreciate the help of Eric J. Wagner and David Galloway in the critical reading of this manuscript.

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Cancer Res 2003;63:6154-6157.

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