Chimeric peptide nucleic acid compounds modulate splicing of the bcl-x gene in vitro and in vivo

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ABSTRACT

Alternative splicing of the bcl-x gene generates two transcripts: the anti-apoptotic bcl-xL isoform and the pro-apoptotic bcl-xS isoform. The ratio between the two isoforms is a key factor in development and in cancer progression. Here, we show that a short antisense chimeric peptide nucleic acid (PNA) oligonucleotide conjugated to a polypeptide containing eight Ser-Arg repeats (SR)₈ can modulate splicing of bcl-x both in vitro and in vivo and induces apoptosis in HeLa cells. The PNA-SR oligo was targeted to a region of bcl-x that does not contain splicing regulatory sequences and was able to override the complex network of splicing enhancers and silencers that regulates the ratio between the two bcl-x isoforms. Thus, PNA-SR oligos are powerful tools that can potentially modulate splice site choice in endogenous genes independent of the presence of other splicing regulatory mechanisms on the target gene.

INTRODUCTION

Analysis of the human genome indicates that at least 65% of human genes are alternatively spliced (1), which contributes significantly to the complexity of the human proteome. The understanding of the mechanisms that regulate alternative splicing and the development of novel technologies to modulate splicing patterns thus have great therapeutic potential in the treatments of cancer, as well as genetic and infectious diseases.

In tumors, the alternative splicing pattern of several genes, including bcl-x, can undergo dramatic changes. Bcl-x is a member of the bcl-2 family of apoptosis regulators and alternative splicing of the bcl-x pre-mRNA generates two main transcripts, a long (bcl-xL) and a short (bcl-xS) isoform, that encode proteins with antagonistic functions (2). Bcl-xL inhibits apoptosis through heterodimerization with pro-apoptotic proteins (3), whereas bcl-xS has pro-apoptotic properties. A proper expression ratio between isoforms is essential in several physiological and developmental processes, such as thymic selection, mammary gland involution and neural reshaping (4,5).

Bcl-xL is over-expressed in numerous types of cancer including myelomas, lymphomas, hepatomas, neuroblastomas and breast cancers (6–10). This over-expression of bcl-xL is associated with decreased apoptosis in tumors, resistance to chemotherapeutic drugs and a poor clinical outcome. Since bcl-xS over-expression can induce apoptosis in tumoral cell lines (11), the ability to alter the bcl-xL/xS ratio thus has great therapeutic potential for the treatment of cancer. Previous work has shown that antisense oligonucleotides targeted to the bcl-xL 5’ splice site can decrease the expression of bcl-xL while increasing bcl-xS expression, thus inducing cell death in response to apoptotic signals (12,13).

However promising, the use of antisense oligonucleotides targeted to splice junctions is not always a suitable approach to alter the splicing pattern of a gene. The blockage of a splice site can result in the activation of a nearby cryptic splice site, which often leads to the appearance of mRNAs which translate for truncated or modified proteins. Additionally, a splice site block can cause unspliced mRNAs to be produced that are subject to rapid nuclear degradation. It is also conceivable that several unrelated genes could be inadvertently targeted by the same antisense oligonucleotide because 5’ splice site sequences have a high degree of conservation.

A different approach to affect splicing regulation, developed by Cartegni and Krainer (14), utilizes a new class of compounds that mimic SR protein function. SR proteins regulate splicing by binding to exonic sequences named exonic splicing enhancers (ESEs) and recruiting components of the splicing machinery to adjacent 5’ and 3’ splice sites (15). The Ser-Arg (SR)-rich domains present at the C-termini of SR proteins mediate such protein–protein interactions. Chimeric antisense peptide nucleic acid (PNA) oligonucleotides (16)
linked to a polypeptide containing 5–10 Ser-Arg repeats have been shown in vitro to activate splicing of substrates derived from the BRCA1 and SMN2 genes (14).

It is unclear whether splicing activation by the chimeric PNA-SR oligos is dependent on the presence of pre-existing splicing regulatory elements or on its targeted position relative to the splice site. In both the SMN2 and BRCA1 substrates, the targeted sequence was a previously characterized ESE, located a few nucleotides downstream of a 3′ splice site, which had been mutated to abolish splicing (14). Furthermore, it is unknown whether the PNA-SR oligos can be delivered to cellular systems to activate splicing in complex endogenous transcripts.

In this work, we utilized an in vitro splicing substrate derived from the bcl-x gene to determine that PNA-SR oligos are suitable tools to activate splicing independent of the presence of pre-existing splicing enhancers and silencers in the target gene sequence. We also determined that a chimeric PNA-SR oligo can efficiently modulate splice site selection in the bcl-x gene in vivo and induce apoptosis in HeLa cells.

MATERIALS AND METHODS

Plasmid constructions
pBK-bclx was generated by amplifying human genomic DNA with the primer pairs MC35.21 (5′-AGATATCTAGATGTCAGAGCACCCGGGAGCTG), MC36.11 (5′-ATAGCGAAATTCCACCCCAACACAGAAGAG), MC36.12 (5′-ATAGCGAAATTCACCCCAACACAGAAGAG) and MC36.13 (5′-TAGATCTCGACACAGTCTAGCTCGTCAGGAACAG). The PCR products were then inserted in pBluescript KS+ under the T7 polymerase promoter. The construct pBK-bclx-PM was generated by PCR-mediated site directed mutagenesis of pBK-bclx utilizing the primers MC35.37 (5′-CACCTTACGGAGATATACTACGAGCTTGGAAAG) and MC35.38 (5′-TGATATCTCTGCTAGGTTGATGTGGAGCTG).

In vitro pre-mRNA splicing assays and preparation of SR proteins
Capped, 32P-labeled run-off transcripts were synthesized by in vitro transcription using T7 RNA polymerase. HeLa cell and S100 extracts were prepared as described previously (17). In vitro splicing reactions were performed in a total volume of 25 μL, containing 15 μL of HeLa cell nuclear extract or S100 as described previously (17). The reaction mixtures were incubated at 30°C for 2 h. RNAs recovered from the reaction mixtures were separated on an 8 M urea–6% polyacrylamide gel and visualized with a Kodak 200R Image Station. Splicing ratios were calculated adjusting for the relative length of the spliced and unspliced PCR products.

Western blot analysis
HeLa cells were transfected as described above and 24 h post-transfection, cells were scraped from six-well plates into the media, washed twice with phosphate-buffered saline and resuspended in 80 μL of SDS–polyacrylamide gel electrophoresis sample buffer. Samples were subjected to electrophoresis on a SDS–12% polyacrylamide gel and transferred to a PVDF membrane (Immobilon™ P, Millipore). Membranes were immunoblotted using a rabbit anti-bcl-x polyclonal antibody S18 (Santa Cruz Biotech) or a rabbit anti-hnRNP H/H′ polyclonal kindly provided by Dr D. L. Black (University of California, Los Angeles) and visualized by chemiluminescence (ECL™-system, Pierce).

Annexin-V/prodipidium iodide staining
HeLa cells were transfected as described above with 5 μM of PNA-(SR)8, PNA oligo control or (SR)8 polypeptide per well. Twenty-four hours post-transfection, cells were stained with Annexin-V and propidium iodide using the Annexin-V-Fluos Staining Kit (Roche) following the manufacturer’s instructions. Annexin-V and propidium iodide staining were then detected by microscopy using a FITC filter (Zeiss inverted microscope-Axiovert 40 CFL).

RESULTS

SF2/ASF and SC35 activate bcl-x splicing
A combination of exonic silencers, exonic enhancers and intronic regulatory elements has been shown to regulate alternative splicing of the bcl-x gene in response to a variety of stimuli (Figure 1A) (20,21). To date, hnRNPs H and F are the only splicing factors found to bind one of those elements to regulate splicing (B2G in Figure 1A) (21).

To gain insight into the regulation of bcl-x splicing, we constructed a minimal bcl-x splicing substrate containing the coding regions of exons 2 and 3 with a shortened intervening intron (Figure 1A). All the exonic splicing regulatory peptides were synthesized and high-performance liquid chromatography purified by Oswald Scientific.

Transfection of PNA-SR oligos and RT–PCR
HeLa cells were maintained at below 80% confluence in D-MEM (Gibco BRL) supplemented with 5% calf serum and antibiotics. Cells were seeded 24 h before treatment at 40% confluence in a 24-well plate. At the time of transfection, growth media was removed and replaced with fresh media not containing serum or antibiotics. In addition, a mixture of Lipofectamine 2000 (Invitrogen) and oligos was added to the cells. After 5 h, growth media containing the transfection mixture was replaced with fresh media containing serum and antibiotics. Cells were harvested 48 h after transfection. Total RNA was extracted with Trizol (Invitrogen), reverse transcribed utilizing a poly(dT) oligonucleotide, and PCR amplified with primers MC21.50 (5′-GCGTCAGGAGGCAGGCAGAC) and MC36.13 for 32 cycles. PCR products were visualized and quantified with a Kodak 200R Image Station. Splicing ratios were calculated adjusting for the relative length of the spliced and unspliced PCR products.
sequences previously characterized (20,21) are present in the pBK-bclx splicing substrate, whereas only part of the Intronic responsive element (IRE in Figure 1A) is present (22). Addition of further intronic sequences to include the entire IRE did not alter the bcl-xL/bcl-xS splicing ratio (data not shown).

In vitro splicing in HeLa nuclear extracts of the pBK-bclx substrate generated two spliced products corresponding to the bcl-xL and bcl-xS isoforms. The 12.8 ratio between the two isoforms (Figure 1B) is remarkably similar to the 11.4 bcl-xL/bcl-xS ratio of the endogenous gene in HeLa cells (Figure 1C).

Members of the SR protein family are arguably the best characterized regulators of splicing. Single SR proteins have been shown to have similar, yet distinct substrate specificities and thus can differentially promote splicing of a given substrate or splice site. Nevertheless, SR proteins also operate as a constitutive part of the splicing machinery and are essential for the splicing of all transcripts (15,23,24). To determine whether specific SR proteins were involved in the splicing regulation of the bcl-x gene, the pBK-bclx splicing substrate was incubated in HeLa S100 cytoplasmic extracts complemented with single SR proteins. S100 extracts are depleted of SR proteins, thus splicing reactions performed with such extracts are not efficient. However, complementation with SR proteins has been shown to restore the ability of S100 extracts to activate splicing (23,25). Splicing of pBK-bclx was only slightly activated by complementation by SRp40, SRp55 or SRp75 (Figure 2, lanes 6–8). In contrast, complementation by a mixture of all SR proteins, SF2/ASF or SC35 activated splicing of the pBK-bclx substrate to levels similar to that obtained with nuclear extract (Figure 2, lanes 3–5).

Addition of SF2/ASF or SC35 to the splicing deficient S100 extracts did not significantly alter the bcl-xL/bcl-xS ratio, 14.2 and 11.8, respectively (versus 12.8 when incubated with nuclear extract). This indicates that SF2/ASF and SC35 may play a
20 Regulatory sequences that either upregulate or downregulate splicing at one of the two alternative 5′ splicing domain (Figure 3B, lanes 5–7) or by the (SR)8 peptide required. Single SR proteins were normalized in activity so that they had equivalent function in activation of splicing of a substrate containing the first intron of the human β-globin gene when added to a S100 extract (data not shown). Splicing ratios and error bars have been obtained from three independent experiments.

To enhance the splicing of the bcl-x gene, a chimeric PNA oligo tethered to eight Ser-Arg (SR) repeats was made complementary to a site 20–31 nt upstream of the bcl-x 5′ splice site (Figure 3A). The addition of the PNA-SR oligo to the in vitro splicing reactions in HeLa nuclear extract caused a dramatic dose-dependent switch in the bcl-xL/XS ratio from 12.8 to 0.3 (Figure 3B, lanes 1–4). The switch in isoform ratio occurred at a compound concentration of 0.5–5 μM. Splicing was unaffected by a control PNA oligo lacking the (SR)8 domain (Figure 3B, lanes 5–7) or by the (SR)8 peptide alone (Figure 3B, lanes 8–9), demonstrating that both the antisense PNA targeting domain and the SR domain were required.

Regulatory sequences that either upregulate or downregulate splicing are often found in close proximity to the splice site they regulate (26). The absence of an effect by the PNA oligo control indicates that no other regulatory factors bind in the same region targeted by the antisense oligonucleotide. Nevertheless, to verify that no regulatory sequence is present in the region targeted by the PNA-SR antisense oligo, we mutated the splicing substrate (pBK-bclx-PM in Figure 3A). The wild-type pBK-bclx and mutant pBK-bclx-PM substrates splice with similar efficiency (12.8 and 13.7, respectively), thus confirming that no ESE or ESS sequences are present in the region targeted (Figure 3B). Additionally, the PNA-SR oligo, the PNA alone and the (SR)8 peptide alone all had no effect on the splicing of the mutant pBK-bclx-PM substrate (data not shown).

The PNA-SR oligo can modulate bcl-x splicing in vivo

Since the chimeric PNA-SR oligo was able to dramatically and specifically switch splicing to the bcl-xS isoform in vitro, we investigated the possibility that this oligo would also alter the bcl-xL/XS ratio in HeLa cells. The PNA-SR oligo was transfected into HeLa cells by cell scraping, spontaneous uptake and Lipofectamine. Transfection by Lipofectamine caused a dose-dependent switch in the bcl-xL/XS ratio, at both the RNA and protein levels, when cells were transfected with a 10 μM solution of the PNA-SR oligo (Figure 4A, lanes 2–8 and Figure 4B, lanes 1–3), while no change was observed when cells were transfected by spontaneous uptake or cell scraping at concentrations up to 50 μM (data not shown). Using Lipofectamine, the bcl-xL/XS mRNA ratio decreased from 11.4 in untreated cells to 4.9 when the PNA-SR oligo was added to a final concentration of 50 μM. The PNA-SR oligo effect on the bcl-xL/XS ratio was highly specific since neither the PNA oligo alone nor the (SR)8 peptide alone affected bcl-x splicing (Figure 4A, lanes 5–8 and Figure 4B lanes 4–7). This is the first demonstration that PNA-SR oligos can modulate splicing in vivo.

Modulation of splicing by PNA-SR oligos induces apoptosis in HeLa cells

The short isoform (bcl-xS) of the bcl-x gene is known to have pro-apoptotic properties. To study the effect that alteration of the bcl-xL/XS ratio by the PNA-SR oligo had on the transfected cells, we utilized Annexin-V and propidium iodide staining to detect apoptosis-mediated cell death and necrosis. During apoptosis, cells break up the phospholipid symmetry of their plasma membrane and expose phosphatidyserine on the external side of the lipid bilayer. Annexin-V preferentially binds to negatively charged phospholipids like phosphatidyserine and thus can be used to detect cells undergoing apoptosis. Following transfection with the bcl-xS-specific PNA-(SR)8 oligo, cells showed a much higher level of apoptosis than cells that were transfected with the PNA alone or (SR)8 peptide control (Figure 5). The increased level of apoptosis was evident 24 h after transfection, whereas no increase in the level of necrotic cells was detected (data not shown). The chimeric PNA-SR oligo thus specifically triggers apoptosis in HeLa cells.

DISCUSSION

Utilizing the bcl-x gene as a model, we have shown that chimeric antisense PNA oligos conjugated to an SR domain can...
modulate splicing patterns independent of the existence of an SR-dependent-exonic splicing enhancer and that they can be efficiently delivered in vivo to trigger apoptosis in HeLa cells. SR proteins are important regulators of alternative splicing and two members of this family, SF2/ASF and SC35, up-regulate splicing of the bcl-x substrate. Nevertheless, SF2/ASF and SC35 did not promote specific splice site selection in this alternative splice site system.

Although SR proteins do not appear to be involved in regulating splice site choice in bcl-x, we showed that a chimeric oligo mimicking SR protein activity (PNA-SR) targeted upstream of the bcl-x 5′ splice site did redirect the splicing machinery, inducing a dramatic switch in the ratio between the bcl-xL and bcl-xS isoforms. The use of PNA-SR compounds was developed previously by Cartegni and Krainer (14) who utilized in vitro splicing substrates derived from the SMN2 and BRCA1 genes. In that study, the PNA-SR compounds were targeted to a sequence immediately downstream of the 5′ splice site to restore inclusion of a skipped exon (14). In contrast, the sequence we targeted in bcl-x is located upstream of a 5′ splice site and the PNA-SR oligo causes that splice site to become preferentially utilized over a downstream 5′ splice site. The different kinds of splicing regulation induced, as well as the different positioning of the oligos in these systems, underscore the versatility of this technique.

Control PNA oligos not carrying the SR peptide have been shown to activate splicing when targeted to the BRCA1 and SMN2 substrates, although requiring a PNA oligo concentration 5–10 times higher to achieve comparable effects to the PNA-SR oligo (14). For bcl-x, we found that the PNA alone control oligo had no effect in vitro, even at a concentration of 5 µM. The discrepancy in the effect of the control PNA oligos between the bcl-x and the BRCA1 and SMN2 substrates can be explained, at least for the SMN2 substrate, with the fact that

Figure 3. A PNA oligo conjugated to an SR domain switches splicing to bcl-xS in vitro. (A) Schematic representation of the PNA-SR oligonucleotide, the location of its target sequence on the bcl-x transcript and likely mechanism of activation of the -xS splice site through recruitment of U1 snRNP. The mutations in the pBK-bclx-PM substrate are indicated. (B) In vitro splicing reactions. Increasing amounts of PNA-(SR)8 oligo, the control PNA oligo or the SR peptide alone were added to in vitro splicing reactions containing HeLa nuclear extract and the pBK-bclx substrate. The splicing profiles of pBK-bclx and pBK-bclx-PM are compared (lanes 10–11). Splicing ratios and error bars have been obtained from three independent experiments.
The binding of the control PNA to the SMN2 substrate masks the binding site for hnRNP A1, a negative regulator of splicing (27), thus de-repressing splicing.

Transfection of antisense oligonucleotides to block the use of the bcl-xL 5′ splice site has been shown to efficiently alter the bcl-xL/bcl-xS splicing ratio at a concentration of 1 µM (12,13). Additionally, antisense oligos that contain protein binding sites as part of a non-hybridizing 5′ tail targeted upstream of the bcl-xL 5′ splice site have been shown to alter the splicing ratio in the nM range (28). Here, we showed that a detectable change in the bcl-xL/bcl-xS ratio in vivo is observed upon transfection of cells with the PNA-SR oligo at a concentration higher than 10 µM. Rather than blocking the bcl-xL 5′ splice site as previous studies have done, we activated splicing to bcl-xS and thus a high concentration of PNA-SR oligo may be needed to overcome the active mechanism repressing use of the bcl-xS 5′ splice site. Furthermore, it is possible that the chimeric PNA-SR oligonucleotide cannot efficiently cross the cellular and/or the nuclear membranes. Conjugation of the PNA-SR oligo with translocation peptides or to several positively charged residues (29) may be used to facilitate the absorption through the membranes and the nuclear localization of these oligos.

Nevertheless, our data shown in Figure 5 indicate that the PNA-SR oligo can induce apoptosis in HeLa cells by altering the bcl-xL/bcl-xS ratio. Apoptosis is detected by 24 h after transfection of the cells with a 5 µM solution of the PNA-SR oligo. This concentration of PNA-SR oligo is much lower than the one utilized to observe a marked shift in the bcl-xL/bcl-xS ratio at the RNA and protein levels (Figure 4). It is possible that only a sub-population of cells is transfected with high efficiency by the PNA-SR oligo, and thus the non-transfected cells dilute the effect of the splicing ratio switch. Furthermore, cells that are transfected with high efficiency presumably undergo apoptosis and degrade their mRNAs and proteins at much higher levels than the untransfected healthy cells, thus interfering with a direct analysis of the bcl-xL/bcl-xS ratio switch.

PNAs have a neutral peptide-like backbone, form highly stable and specific base pairing with RNA, are resistant to nucleases, do not trigger RNase H activity and can be easily conjugated with any polypeptide (16). Furthermore, PNA oligos have been successfully delivered into cells in culture and into animal tissues (30). All these characteristics make these oligos attractive for therapeutic applications.
compounds ideal for their use as therapeutic agents. Not only can PNA-SR oligos modify the expression of single protein isoforms, but they also have the potential to generate novel mRNAs coding for new proteins. This would further increase the diversity of the proteome and possibly lead to new therapeutic tools.

In particular, it is conceivable that PNA-SR oligos could be targeted to activate cryptic splice sites that closely match the consensus sequence but are normally not used in vivo. The splicing machinery often does not recognize such splice sites because splicing enhancers within close proximity are often required for a splice site to be active. Accordingly, we have previously shown that even a perfect consensus 5' splice site cannot splice without the presence of a nearby ESE in a viral transcript (31). Targeting a PNA-SR oligo in proximity of a cryptic splice site could activate such site, thus generating novel gene products.

An example for such applications could be the bcl-2 gene, an inhibitor of apoptosis. Despite its high homology at the nucleotide level with bcl-x, the bcl-2 gene is not alternatively spliced into a short and a long isoform like bcl-x. The bcl-xS 5' splice site sequence AG/GUAGUG is similar to the corresponding sequence present in the bcl-2 gene CG/GUGGUG that is never utilized, but is close to the consensus 5' splice site AG/GURAGU. Therefore, it may be possible to use a PNA-SR oligo targeted to that region of bcl-2 to create a novel bcl-2 isoform that encodes a protein that is likely to have pro-apoptotic properties. PNA-SR oligos have the potential to change not only known splicing patterns, but also to create novel gene products in cellular and possibly animal systems.

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