Rescue of a human mRNA splicing defect by the plant cytokinin kinetin

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Received October 16, 2003; Revised and Accepted December 17, 2003

The defective splicing of pre-mRNA is a major cause of human disease. Exon skipping is a common result of splice mutations and has been reported in a wide variety of genetic disorders, yet the underlying mechanism is poorly understood. Often, such mutations are incompletely penetrant, and low levels of normal transcript and protein are maintained. Familial dysautonomia (FD) is caused by mutations in $\textit{IKBKAP}$, and all cases described to date involve an intron 20 mutation that results in a unique pattern of tissue-specific exon skipping. Accurate splicing of the mutant $\textit{IKBKAP}$ allele is particularly inefficient in the nervous system. Here we show that treatment with the plant cytokinin kinetin alters splicing of $\textit{IKBKAP}$. Kinetin significantly increases inclusion of exon 20 from the endogenous gene, as well as from an $\textit{IKBKAP}$ minigene. By contrast the drug does not enhance inclusion of alternatively spliced exon 31 in $\textit{MYO5A}$. Benzyladenine, the most closely related cytokinin, showed a similar but less dramatic effect. Our findings reveal a remarkable impact on splicing fidelity by these small molecules, which therefore provide new tools for the dissection of mechanisms controlling tissue-specific pre-mRNA splicing. Further, kinetin should be explored as a treatment for increasing the level of normal IKAP in FD, and for other splicing disorders that may share a similar mechanism.

INTRODUCTION

The recent sequencing of the human genome has led to surprisingly low estimates of the number of human genes, in contrast to the significantly higher estimates that were previously based on expressed sequence tags in the public databases (1,2). This apparent disparity suggests a major role for alternative splicing in creating genetic complexity, and has brought the study of splicing regulation to the forefront of molecular genetics. Indeed, the misregulation of this process is a common cause of genetic disease as ~15% of all known human mutations result in defective pre-mRNA splicing (3). The splicing reaction is carried out by the spliceosome, which consists of five small nuclear ribonucleoprotein complexes, or snRNPs, and as many as 50–100 non-snRNP proteins (4,5). Spliceosome assembly also requires SR proteins, a family of sequence specific splicing factors that have one or two RNA-recognition motifs followed by an arginine/serine rich domain. Numerous SR proteins have been identified to date, and they have diverse roles in constitutive and alternative splicing (6). Members of the heterogeneous nuclear ribonucleoprotein (hnRNP) factor family also play a role in splicing by promoting the use of distal 5' splice sites, which can affect both exon skipping as well as exon inclusion (7,8). Several SRs and hnRNPs have been shown to modify the splicing pattern of human transcripts carrying splice mutations. HnRNP A1 and ASF/SF2, and their adenoviral analogs E4-ORF6 and E4-ORF3, have been shown to modulate the splicing pattern of $\textit{CFTR}$ alleles that carry cystic fibrosis causing mutations (9). Htra2-β1, an SR-like splicing factor, dramatically enhanced the inclusion of exon 7 in $\textit{SMN2}$ transcripts resulting in an increased amount of full-length protein (10). Further, it has been demonstrated that chemical compounds and drugs can also alter splicing. Sodium butyrate, aclarubicin, sodium vanadate and valproic acid have all been shown to increase the amount of exon 7 containing SMN protein in spinal...
muscular atrophy (SMA) lymphoid cell lines, and treatment of SMA-like mice with sodium butyrate promoted increased expression of SMN protein in motor neurons and resulted in significant improvement of clinical symptoms (11–15). Familial dysautonomia (FD, Riley–Day syndrome, hereditary sensory and autonomic neuropathy type III, MIM 223900) is a recessive disorder that has a remarkably high carrier frequency of 1 in 30 in the Ashkenazi Jewish population (16). The clinical spectrum of FD includes gastrointestinal dysfunction, abnormal respiratory responses to hypoxic and hypercarbic states, gastroesophageal reflux, vomiting crises, lack of overflow tears, profuse sweating and postural hypotension (17). Three FD mutations have been identified in the Iκ-B kinase (IKK) complex-associated protein (IKBKAP), IVS20*+6T→C, which leads to variable, tissue-specific skipping of exon 20 (Fig. 1A), R696P and P914L (18–20). All FD patients tested to date carry at least one IVS20*+6T→C mutation, with more than 99.5% being homozygous, and the remainder being heterozygous with either R696P or P914L on the alternate allele. This suggests that production of wild-type IKBKAP from the mutant allele is required for viability, and that the tissue-specific variability of IKAP results in the distinctive FD phenotype. IKAP has been reported to be both a member of the Elongator complex (21), which plays a role in transcriptional elongation, and a c-Jun N-terminal kinase (JNK)-associated protein (22), which is involved in the mammalian stress response. We have reported previously that all FD tissues tested express both wild-type (WT) and mutant (MU) IKBKAP mRNA (20,23). Accurate measurement of the ratio of the two mRNA species using both densitometry and real-time quantitative PCR has revealed that the levels of WT IKBKAP mRNA vary between tissues and are lowest in central and peripheral nervous systems (23). This leads to a drastic reduction in the amount of IKAP protein in these tissues. Our observations suggest that the relative inefficiency of WT IKBKAP mRNA production from the mutant alleles in the nervous system underlies the selective degeneration of sensory and autonomic neurons in FD. Given that neuronal degeneration continues throughout life in FD patients, a promising route toward effective therapies may come from enhancement of correct mRNA splicing in order to increase cellular levels of IKAP.

To this end, we devised an assay that provides stable and consistent measurement of the ratio of WT: MU FD IKBKAP mRNA splice products in lymphoblast cell-lines (23). This assay was employed as part of the Neurodegeneration Drug Screening Consortium (24) to identify compounds that increase the relative production of WT mRNA. Here we report that kinetin, a plant cytokinin, dramatically increases the amount of wild-type IKBKAP mRNA in FD cell lines. Further, we show that this leads to a significant increase in the amount of IKAP protein and suggest that kinetin, an FDA approved drug, should be tested as a possible treatment for FD.

RESULTS

The initial screen was carried out using a single, standard FD lymphoblast cell line that was assayed after 72 h in the presence of 10 μM test drug, using compounds dissolved in DMSO. The major FD splicing mutation results in production of a mutant IKBKAP mRNA that is missing exon 20 (Fig. 1A). We screened a panel of 1040 bioactive compounds (NINDS Custom Collection, MicroSource Discovery Systems), most of which have been approved for use by the Food and Drug Administration (FDA). Prior to initiating the screen, we confirmed that DMSO had no effect on IKBKAP splicing and was not toxic to the cells at the test concentration of 0.1%. One compound, kinetin (6-furfurylaminopurine) (Fig. 1B), dramatically enhanced correct splicing in FD cells at the test concentration of 10 μM (Fig. 1C).

We next tested concentrations of kinetin up to 100 μM and observed significant enhancement of WT IKBKAP production with increasing concentration in two independent FD lymphoblast lines (Fig. 2A). The increase in WT: MU mRNA was mirrored by a significant increase in production of IKAP protein (Fig. 2B), confirming the improved production of functional WT mRNA. Quantitative analysis of the western blot in Figure 2B shows a 200% increase in IKAP production with each 10-fold increase in kinetin concentration. To avoid DMSO toxicity at higher concentrations, we purchased kinetin from an independent source (Sigma) dissolved in water. Real-time quantitative PCR analysis showed that the WT: MU IKBKAP mRNA ratio increased steadily from ~1:1 in untreated cells to 12:1 in cells treated with 400 μM kinetin, the maximum dose tested due to the appearance of some cellular toxicity (Fig. 2C). By contrast, total IKBKAP mRNA was relatively unaffected, with a slight increase (~1.5-fold) being detected only at doses higher than 50 μM (data not shown).

Kinetin belongs to the family of N6-substituted adenine derivatives known as cytokinins, or plant growth factors, that also includes zeatin, benzyladenine and 2iP (Fig. 1B). In our FD lymphoblast assay, neither zeatin nor 2iP had any effect on WT : MU IKBKAP ratio. Benzyladenine did enhance inclusion of exon 20 in the IKBKAP transcripts ~2-fold by 100 μM, a less dramatic effect than kinetin (data not shown). The fact that only kinetin and benzyladenine alter IKBKAP splicing suggests that the nature of the adenine N6-linked modification plays a role in this function.

The experiments described above were all performed after 72 h of treatment with kinetin. In order to define how quickly kinetin alters IKBKAP splicing, we performed a time-course at 50 μM kinetin (Fig. 2D). The increase in the WT : MU ratio was seen at 1 h, was maximal by 8 h, and was maintained for at least 72 h in culture without kinetin replenishment. The consistency of the effect was tested by treating lymphoblast lines from nine FD patients with 50 μM kinetin for 24 h. All nine lines showed an increase in WT IKBKAP mRNA with WT: MU ratios ranging from 1.3 to 1.9 in untreated cells to 5.6–8.6 in kinetin treated cells. Kinetin was also effective in FD fibroblast lines which exhibited a dose-dependent increase in WT: MU IKBKAP ratio similar to that seen in lymphoblasts (data not shown).

To explore whether the effect of kinetin was specific to IKBKAP exon 20 or generally increased inclusion of alternatively spliced exons, we assayed splicing of the MYO5A gene (Fig. 3A). MYO5A expresses multiple isoforms in fibroblasts and lymphoblasts, one of which is generated by skipping of exon 31 (25). We designed primers flanking exon 31 and examined the ratio of the two MYO5A isoforms in nine FD lymphoblast cell lines following treatment with 50 μM kinetin.
for 72 h. No significant difference was observed in the ratio of the two isoforms between the treated and untreated samples, with average transcript ratios of 2.33 and 2.46, respectively (Fig. 3B).

The IVS20+6T→C mutation in IKBKAP leads to a frameshift and a premature termination codon in exon 21, which is expected to target the mutant message for decay via the nonsense-mediated mRNA decay (NMD) pathway (26). While we hypothesize that kinetin is acting directly on IKBKAP splicing, it is possible that an increase in NMD of the mutant transcript could result in the observed changes in WT:MU ratio. Therefore, we exposed kinetin-treated FD cells to cycloheximide, a translation inhibitor, in order to halt NMD of the mutant transcript (27). As shown in Figure 4, cycloheximide increases the MU transcript and decreases the WT:MU ratio as expected. However, kinetin significantly alters the WT:MU ratio even when the NMD pathway is inhibited, an observation consistent with the hypothesis that kinetin is acting on IKBKAP splicing.

In order to better understand the mechanism of action of kinetin on IKBKAP splicing, we created both FD and WT minigene constructs containing the genomic sequence spanning exons 19–21 (Fig. 5A). These constructs were transfected into HEK293 cells and RNA amplified using vector-specific primers to avoid amplification of the endogenous IKBKAP message. Evaluation of the transcripts produced from the WT construct showed that although the WT transcript is the predominant product, trace levels of exon 20 skipping do occur even in the absence of the FD mutation. Introduction of the FD mutation into the construct increased exon 20 skipping as predicted. Interestingly, treatment with kinetin enhanced inclusion of exon 20 not only in the FD construct, but also in the WT construct (Fig. 5B). In fact, a MU-specific PCR assay that utilizes a primer spanning the junction of exons 19 and 21 (23) revealed that exon 20 skipping is completely corrected by kinetin treatment in transcripts generated from the WT construct (Fig. 5C). These findings indicate that kinetin’s ability to enhance splicing efficiency is not dependent on either
the presence of the FD mutation or the wider regulation of IKBKAP transcription or IKAP protein production, and is likely due to specific sequence elements in the region of exon 20 that function to regulate splicing of this particular exon.

**DISCUSSION**

Mutations in the splice donor site that alter either the fifth or sixth base position, and might therefore be expected to lead to variable exon skipping, have been reported for over 30 different genetic disorders. FD is unusual among the common Ashkenazi Jewish disorders in that it is caused by only three mutations, the most frequent of which accounts for >99.5% of all FD chromosomes. This IVS20+6T→C mutation is present on at least one chromosome in all FD patients identified to date. Given that FD is universally associated with one particular 'partial' splicing mutation that results in subtle variability in IKAP levels suggests that tissue-specific splicing regulation underlies the development of the FD phenotype.

In this study we demonstrate that kinetin can significantly increase the level of full-length IKAP protein in FD cells. This is exciting since even subtle changes in the level of functional protein have been shown to have a drastic effect on phenotype. For example, mutations in ATP7A are responsible for both Menkes disease (MD), a lethal disorder of copper metabolism, and occipital horn syndrome (OHS), a milder disorder characterized by connective tissue abnormalities. MD is caused by mutations in the invariant donor splice site of exon 6 that lead to complete exon skipping, while OHS is the result of a mutation at bp 6 of the same donor splice site that leads to incomplete exon skipping, resulting in the milder phenotype (28). The level of functional protein in OHS is only 2–5% of normal, but sufficient to significantly alter the resulting

![Figure 2](image-url). The action of kinetin on IKBKAP splicing is dose and time dependent and results in an increase of IKAP protein in FD lymphoblast cell lines. (A) RT-PCR demonstrating increasing WT:MU IKBKAP ratios as a result of increased kinetin concentration. Sizes of the WT and MU bands are shown on the right, and the IDV ratios are shown beneath each lane. Adjacent lanes under each treatment represent two independent FD cell lines. (B) Western blot probed with an IKAP monoclonal antibody showing that increasing concentrations of kinetin result in an increase in IKAP protein production in FD cells. The lower panel shows the same blot probed with an IDE antibody as a protein loading control. (C) Graph of WT:MU IKBKAP ratios at increasing kinetin concentrations determined by QPCR of FD cells (treated with kinetin in water in duplicate and each amplified in triplicate) showing that higher doses of kinetin continue to increase WT:MU IKBKAP. Examination of (A) shows that at kinetin concentrations of 100 μM the MU band is barely discernable using densitometry, therefore QPCR was used for this study. (D) Graph of WT:MU ratios generated using IDV values following culturing of FD cells in 50 μM kinetin for increasing lengths of time. All treatments were performed in duplicate and average data points are plotted.
expression or activation can mediate both constitutive and alternative splicing (8,9,31,32). Their direct positive effect on exon inclusion when disease-causing mutations are present has been shown for both CFTR and SMN2 transcripts (9,10). In addition, immunofluorescence experiments demonstrate that aclarubicin induces a redistribution of SR proteins (12). Very recently, it has been reported that (−)−epigallocatechin gallate (EGCG) can increase correct IKBKAP splicing in FD cells (33). EGCG, an antioxidant found in green tea, is known to down-regulate the expression of hnRNP A2/B1 (34), which may explain its effect on IKBKAP splicing. Kinetin is currently marketed as an anti-aging ingredient in skin treatments due to its ability to ameliorate aging characteristics in cultured human fibroblasts (35), possibly through anti-oxidant activity (36). Our demonstration that kinetin can increase correct splicing of the control IKBKAP minigene construct suggests that its function is not dependant on the IVS+6 mutation, but rather linked to particular sequence elements that control IKBKAP exon 20 splicing. This finding, combined with the studies cited above, suggests that kinetin likely influences the activation or cellular localization of specific spliceosomal proteins. The effect of EGCG on WT IKBKAP expression and cellular IKAP levels is not as dramatic as that of kinetin, but it will be interesting to compare their activities directly and assess their therapeutic value when used together. Further studies into the mechanism of action of these compounds will no doubt lead to a better understanding of a fundamental regulatory process controlling tissue-specific gene expression. In the meantime, they may be useful not only in the treatment of FD, but also of many other disorders caused by splicing mutations under similar regulatory control.

MATERIALS AND METHODS

Patient cell lines

Patient lymphoblast cell lines previously established in our laboratory by EBV transformation were utilized in this study (20,37). We have current Institutional Review Board approval for the establishment and use of these lines through New York University Medical Center, Massachusetts General Hospital, and Harvard Medical School. Two fibroblast cell lines were used in the current study, GUS12829 was established in our laboratory and GM02343 was purchased from the Coriell Cell Repository.
Drug screen

The panel of 1040 independent compounds used was specifically designed for the NINDS sponsored Neurodegeneration Drug Screening Consortium (MicroSource Discovery Systems). A single FD lymphoblast cell line was cultured in 24-well format and treated with 10 mM compound dissolved in DMSO for 72 h. RNA was extracted using Tri-Reagent™ (Molecular Research Center) and reverse transcription and IKBKAP PCR was performed as previously described (23).

Cell culture and treatment

FD lymphoblast lines were grown in RPMI-1640 and primary fibroblast lines in Dulbecco’s modified Eagle’s media (DMEM) with Earle’s balanced salts. Both media were supplemented with 2 mM L-glutamine and 1% penicillin/streptomycin and either 10% (lymphoblast) or 20% (fibroblast) fetal bovine serum (Invitrogen). Kinetin was obtained from both MicroSource Discovery Systems and Sigma and dissolved at 10 mM in either DMSO or water. FD cells were cultured in the presence of kinetin for 72 h except where noted. Benzyladenine, zeatin, and 2iP were obtained from Sigma and dissolved in DMSO at 10 mM. These compounds were tested at concentrations of 1, 10, 50 and 100 mM. Cycloheximide was obtained from Sigma and dissolved at 10 mg/ml in DMSO. Cells were treated with 100 μM kinetin for eight hours, then 50 μg/ml cycloheximide was added to the cultures for 4 h (27).

Determination of IKBKAP transcript ratios

The ratio of WT:MU IKBKAP transcripts was determined following amplification by fractionation on 1.5% agarose gels stained with ethidium bromide (23). Each gel band was assessed using an Alpha 2000™ Image Analyzer and software coupled with automatic background subtraction (Alpha Innotech Corporation). WT : MU ratios were obtained using the integrated density value (IDV) for each band (23). Real-time quantitative PCR (QPCR) was performed as described using primers specific to WT IKBKAP; MU IKBKAP, and 18S ribosomal RNA (23). In order to assess total IKBKAP expression we used the following primers: EX3F—5'-TCAGGACTTGCTGGATCAGGA; and EX4R—5'-CCACTGGCTACACTCCCTTCT located in IKBKAP exons 3 and 4 and an exon 3 TaqMan probe: TCTG-GAGACGTACATCGAGTCTCAGC. For the Myosin VA assay, we designed primers that flank the alternatively spliced exon 31 in order to assess transcript levels of the two isoforms. Primer sequences were as follows: MYO-F—GAA TAC AAT GAC AGA TTC CAC; MYO-R—CAG GCT GGC CTC AAT TGC. Following reverse-transcription and PCR, products were separated on a 1.5% agarose gel and IDV ratios determined as described above.

Western blot analysis

Patient lymphoblast cell lines were treated with increasing concentrations of kinetin for 72 h. Extracted protein was run on a 6% polyacrylamide gel. The samples were transferred to nitrocellulose and stained with Ponceau-S to visualize protein loading. IKAP was detected using a monoclonal antibody raised against amino acids 796–1008 of IKAP (BD Bioscience) that detects the 150 kDa full-length IKAP protein. The same blot was probed with an antibody to insulin-degrading enzyme (IDE) as a protein loading control. Bands were quantitated using an Alpha 2000™ Image Analyzer.

In vivo IKBKAP minigene splicing assay

IKBKAP genomic DNA was amplified from an unaffected and an FD individual using primers in exon 19 and 21: EX19F—
REFERENCES


ACKNOWLEDGEMENTS

We wish to thank all our FD patients and their families for their continued participation in our studies. This work was supported by grants from the Dysautonomia Foundation Inc. (S.A.S. and J.F.G.), the National Institute of Neurological Disorders and Stroke (S.A.S.), and the Harvard Center for Neurodegeneration and Repair (S.A.S.).


