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ZINC FINGER PROTEINS: EPIGENETIC BREAKTHROUGHS AND POSSIBLE TREATMENT OF NEURODEGENERATIVE DISORDERS

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Abstract

Engineered zinc finger proteins (ZFN) that bind specific DNA targets provide the foundation for a new class of technologies that promise significant gains in the development of novel therapeutics and molecular research tools. Individual zinc fingers have been developed *in vitro* to recognize many DNA triplets. When fused to the nonspecific DNA cleavage domain of the FOK1 restriction endonuclease, zinc fingers can direct double strand breaks to disrupt specific genes. Expanded triplet repeats have been identified as the genetic basis for a growing number of neurological disorders. Thus ZFN show promise for the treatment of monogenic disorders by promoting the knockout or correction of specific genes.

Keywords: Zinc finger, DNA, Methylation, Gene, Neurodegeneration

Introduction

Neurons are postmitotic cells that must survive and function properly for the entire lifetime of the organism. Because they cannot be replaced and are subjected to high metabolic stress, mechanisms for coping with damaged molecules may be particularly important in these cells. Indeed, human neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases, involve the abnormal accumulation of damaged proteins (1), and other syndromes, such as ataxia telangiectasia (AT), have been associated with defects in DNA-damage processing (2). Work done by El-Khamisy *et al.* (3) and a more recent study by Ahel *et al.* (4) have revealed that inefficient repair of DNA single-strand breaks (SSBs) can give rise to neurodegenerative disease, in particular, spinocerebellar ataxia with axonal neuropathy-1 (SCAN1) and ataxia oculomotor apraxia-1 (AOA1), respectively. These data provide evidence that non-replicating, post-mitotic neurons are particularly sensitive to the accumulation of DNA SSBs. SSBs, one of the most common lesions formed in chromosomal DNA, are generated by the attack of reactive oxygen species (5) or as natural intermediates during certain DNA transactions, including repair and replication (6). In both instances, SSBs can harbor non-conventional 3' or 5' termini, such as phosphates, phosphoglycolates, or trapped polypeptides, which present obstacles to polymerization and ligation activities. To remove such obstructions, cells undergo SSB repair, a process related to the more classical base excision repair (BER) pathway (7). The SSB repair proteins excise terminal blocking groups, permitting gap-filling synthesis and sealing of the final nick in DNA. Zinc-finger nucleases (ZFN) link a DNA binding domain of the zinc-finger type to the nuclease domain of Fok I and enable the induction of double-strand breaks (DSBs) at preselected genomic sites (4). DSBs closed by the error-prone, nonhomologous end-joining (NHEJ) DNA repair pathway frequently exhibit nucleotide deletions and insertions at the cleavage site. This technology has been applied to introduce knockout mutations into the germ line of rats and zebrafish by the expression of ZFPs in early embryos that target coding sequences (8-11). On the other hand, DNA methylation contributes to cellular reprogramming and views on the function of CpG methylation are changing. In the early 1990s, it was already apparent that promoters with low CpG content did not show a clear relationship between methylation level and expression level (12,13). More recently, analysis of 16,000 promoters in the human epigenome reveals that when CpG density is high there is a greater degree of methylation and this reasonably predicts gene expression. However, when CpG content is low, there is no clear relationship between methylation and expression (14). From the same data set, it is apparent that, whereas DNA methylation is sufficient to inactivate CpG-rich gene promoters, it is not necessary because many hypomethylated CpG-rich promoters are inactive. The associated chromatin structure is implicated as an additional regulatory variable in such circumstances and highlights the important interplay between DNA methylation and histone modifications. Additional proposed functions of CpG methylation include monoallelic expression, parasitic element silencing (12), or changes to the associated chromatin

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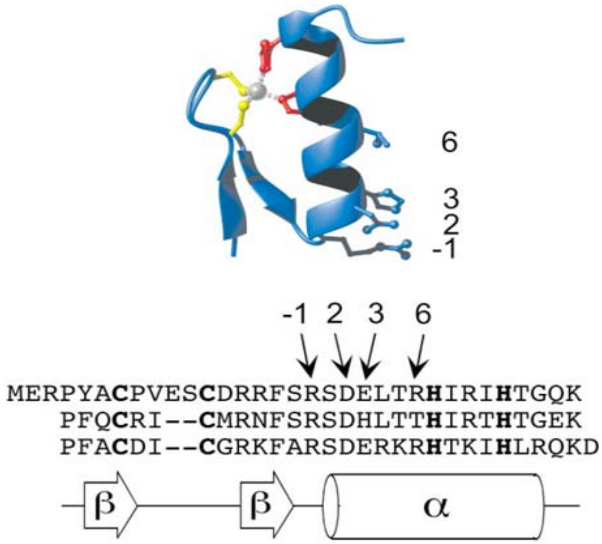
(15). Moreover, some have suggested that many reported instances of a correlation between CpG methylation and expression patterns reflect a consequence of gene transcription on the epigenome and not vice versa. This review examines the importance of these epigenetic breakthroughs for treatment of neurodegenerative disorders by application of ZFPs.

Highly efficient, targeted genome manipulation induced by ZFPs

Gene targeting is a method to repair or inactivate any desired gene of interest. Gene targeting strategies use the introduction of a double-stranded break (DSB) into a genomic locus to enhance the efficiency of recombination with an exogenously introduced homologous DNA "repair template" (Figure 1) (16,17). DSBs can stimulate recombination efficiency several thousand-fold, approaching gene targeting frequencies as high as 50% (18-22). Early experiments utilized highly specific homing endonucleases, enzymes that bind and cleave extended DNA sequences, to introduce DSBs into specific genomic loci (17). To date, however, the use of homing endonucleases to enhance gene targeting has not been demonstrated at an actual endogenous gene locus.

Figure 1: Zif268 and DNA recognition residues



The complex and multisystemic effects caused by defects in basic DNA-repair mechanisms are now well characterized. Research over many years has shown that neurodegenerative defects are often associated with repair syndromes, but it has been very difficult to establish the precise causative links between these processes. However, recent research on the neurodegenerative disorders ataxia with oculomotor apraxia 1 (AOA1) and spinocerebellar ataxia with axonal neuropathy 1 (SCAN1), which are caused by dysfunction of Aprataxin (APTX) and tyrosyl-DNA phosphodiesterase 1 (TDP1), respectively, sheds new light on the underlying basis of DNA repair-associated neurodegeneration (23-25). AOA1 and SCAN1 are autosomal recessive cerebellar ataxias, but in contrast to A-T, their phenotypes are very much restricted to the nervous system. Recent findings indicate that neuronal cell death in AOA1 and SCAN1 is primarily due to the specific proofreading roles that Aprataxin and TDP1 play in the repair of DNA single-strand breaks (SSBs). This unprecedented correlation between molecular defect and neuron-specific impact may provide the missing link that allows us to begin to understand the relationship between neurodegeneration and defective DNA repair.

The zinc-finger domain is required for aprataxin activity

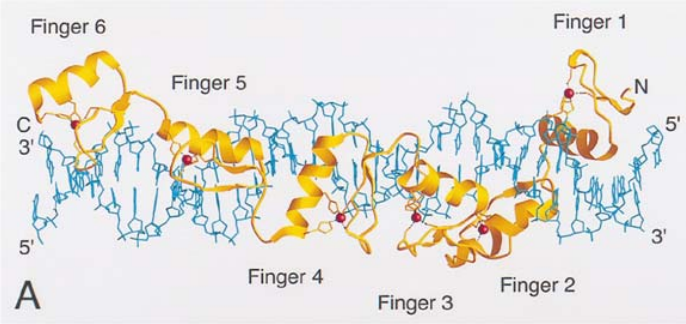
In retrospect, it is surprising that it took so long to uncover a role for Aprataxin as a DNA-specific adenylase. The presence of the N-terminal XRCC1/XRCC4-interacting FHA domain and the C-terminal zinc finger (ZnF) domain provided significant clues to its actions on DNA. Recent studies have shown that Aprataxin binds both double- and single-stranded DNA, in reactions dependent upon the ZnF motif (26). Moreover, the protein exhibits a high affinity for adenylated rather than nonadenylated DNA, and specific complex formation is again dependent upon the zinc-coordinating cysteines C319 and C322 (27). It is likely that Aprataxin uses its general DNA-binding capacity to scan the genome, such that the HIT and ZnF domains may then cooperate to form a high-affinity complex that locks the enzyme onto adenylated

DNA lesions. Consistent with this proposal, the DNA adenylase activity is reduced 100-fold by mutation of the ZnF motif (27). The critical role that the ZnF plays in substrate recognition may explain why mutations that map to the C-terminal side of the catalytic HIT domain confer an AOA1 phenotype that is similar in severity to that caused by mutations within the HIT domain (28).

Selective *in vivo* targeting of DNA methylation by Zinc finger proteins

Mechanistically, one way DNA methylation can lead to transcriptional silencing is by decreasing the binding affinity of a transcriptional activator for its site (29). Although the mechanisms are still being elucidated there is a strong correlation between promoter methylation and gene silencing (30). Thus DNA methylation can be critical in defining the expression state of a gene. The design of multiple zinc-finger with desired specificities is proving a versatile platform for targeting a variety of protein moieties to accessible sites in vivo as shown in Figure 2 (31,32). For instance, engineered zinc-finger proteins have been fused to the catalytic domain of R-Fok1 endonuclease to direct regions (33). Designed ZFP have also been used to target the catalytic domains of the histone methyltransferases G9A and SUV39H1 (34) as well as the VP16 activation domain (35). Leading to repression and activation, respectively, of expression of the human erythropoietin, vascular endothelial growth factor A and other mammanlian genes.

Figure 2: Structure of six fingered TF-DNA complex



The targeting of DMTases by zinc-finger proteins selecte to bind specific ZBS could provide an additional way to down- regulate the expression of desired genes. Moreover, since the DNA methylation state of a given promoter is maintained heritably through DNA replication by endogenous cellular mechanisms, an initial targeting event may be sufficient to establish stable silencing of improperly expressed genes. Therefore, heritable repression could also reduce the amount of treatment necessary to establish the proper regulation of a particular gene. In addition to providing a potentially powerful therapeutic tool, methylation-mediated repression of specific- ally targeted genes could yield an alternative to transgenic knockouts for studying loss-of-function phenotypes. Silencing genes through DNA methylation would be particularly valuable in the case of essential genes where tissue-specific knockouts of function are needed. A ZFN directed at CAG repeat tracts might also prove useful as a therapeutic reagent for treatment of patients. Treatments that can promote the reduction of large CAG repeat tracts, or interrupt them, might form the basis for a future therapeutic approach designed to prevent or delay the onset of large stage neurological disorders caused by expanded CAG repeat tracts (36).

Therapeutic uses of ZFN in neurodegenerative and related disorders

Although ZFPs and ZFNs are still a recent area of research, there are some immediately applicable therapeutic opportunities that have arisen (37). For example, Sangamo biosciences have developed ZFPs fused to the VP16 transcriptional activation domain targeted to the VEGF-A promoter which is currently in Phase 2 trials for diabetic neuropathy and Amyotrophic Lateral Sclerosis (ALS). This drug, which involves direct injection of DNA encoding the ZFPs into the affected site, requires efficient uptake of the DNA and strong expression in the target tissues. In some diseases, it is not genomic DNA that is altered, but mitochondrial DNA. Neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP) and maternally inherited Leigh's syndrome are caused by a single T8993G nucleotide alteration in mitochondrial DNA. A combination of adding a nuclear exclusion sequence and a mitochondrial targeting sequence was shown to be effective in localizing ZFNs to the mitochondria. However, the conventional heterodimeric ZFNs were unsuccessful at binding and/or cleaving the appropriate site in the mitochondrial genome. Therefore a model recently developed shows a novel single ZFN which expresses two Fok1 proteins separated by a flexible

linker, using ZFPs to direct this polypeptide to the T8993G mutation. When expressed in cells expressing copies of both the mutant and WT mitochondrial genome, this *quasi-dimeric* ZFN was able to selectively cleave the mutant copy leading to enrichment of the WT copy, which could be sufficient to reverse phenotypic consequences. ZNF219 is a member of the Krüppel-like zinc finger gene family and is a 77 kDa protein containing nine sets of C2H2 zinc finger structures (38). ZNF219 is widely expressed in many tissues, including the cerebellum, substantia nigra, hippocampus, and cerebral cortex (39, 40). ZNF219 functions as a repressor of transcriptional activation in the promoter of the high-mobility group nucleosome binding domain 1 gene and competes for binding to its target sequence with members of the Sp1 family of transcriptional activators (36). In support of this view, in the luciferase assay, it was observed that no activation for constructs that contained either three or one ZNF219 binding sites. For constructs that contained two ZNF219 binding sites we observed an increase in luciferase activity. Therefore, the exact involvement of ZNF219 in the regulation of SNCA is somewhat unclear, and will require further characterization by deleting individual and multiple sites in the context of the full 1.9 kb construct.

Conclusion

It is becoming clear ever since the discovery of zinc finger proteins by Sir Aaron Klug that they could be critical for possible treatment of neurodegenerative disorders. The advances in understanding epigenetic modifications of DNA has opened new vistas for treating neurodegenerative disorders. Silencing genes through DNA methylation would be particularly valuable in the case of essential genes where tissue specific knock outs of function are needed. Moreover, the combination of strong zinc-finger binding and reduced methyltransferase activity gained from rational mutagenesis, has allowed us to target methylation predominantly to specific DNA sequences without incurring significant background methylation. These enzymes are fully functional in mammalian cells, where the targeted methylation pattern delivered is demonstrated to be inherited through successive cell divisions. In summary, the development of enzymes in which the dominant interaction with DNA is clearly driven by the zinc-finger component. The development of targeted Mtase enzymes and methodologies to analyse their function rapidly and within a relatively complex model system, together with the further characterization of their functional activity *in vivo* in mammalian cells, represents an important step towards the ultimate employment of this class of molecule in both research and therapeutic arenas.

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