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Hybrid spectrin type repeats produced by exon-skipping in dystrophin

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Abstract

Dystrophin is the protein whose defect underlies Duchenne Muscular Dystrophy, DMD, a common (1:3500 male births) and fatal condition in which muscle tissue deteriorates leading to death in the second or third decade of life. Dystrophin is coded for by the largest human gene, and one of the most complex. It is translated from at least 7 distinct promoters, with the largest transcripts (which are the ones involved in DMD) containing 79 exons over >2.5 Mbp [K.F. O'Brien, L.M. Kunkel, Dystrophin and muscular dystrophy: past, present, and future, *Mol. Genet. Metab.* 74 (2001) 75–88, H.M. Sadoulet-Puccio, L.M. Kunkel, Dystrophin and its isoforms, *Brain Pathol.* 6 (1996) 25–35]. Exacerbating this complexity, it has recently been shown that dystrophin is subject to extensive alternative RNA processing, potentially producing a wide variety dystrophin variants [M. Sironi, R. Cagliani, U. Pozzoli, A. Bardoni, G.P. Comi, R. Giorda, N. Bresolin, The dystrophin gene is alternatively spliced throughout its coding sequence *FEBS Lett* 517 (2002) 163–166]. The structure of the dystrophin protein is highly modular, with the most common module being a motif termed the spectrin type repeat, or STR, of which there are 24. Each STR is roughly coded for by two exons, and the most common type of multiple exon-skipping events start and end at introns in the middle of STRs [R.G. Roberts, A.J. Coffey, M. Bobrow, D.R. Bentley, Exon structure of the human dystrophin gene *Genomics* 16 (1993) 536–538, M. Koenig, L.M. Kunkel, Detailed analysis of the repeat domain of dystrophin reveals four potential hinge segments that may confer flexibility, *J. Biol. Chem.* 265 (1990) 4560–4566]. This would produce fractional STR modules, however, the concept of STRs as proteins domains makes the viability of such fractional motifs questionable. However, certain of these events produce pairs of potentially complementary fractional domain that might reassemble into a hybrid STR motif. We have constructed model fragment corresponding to one such exon-skipping event, and show that the hybrid STR so produced is viable, and furthermore that some of the properties of the protein containing it differ substantially of the native, un-skipped parent.

Keywords

Dystrophin; Spectrin-type-repeat; Domain; Hybrid-domain; Exon skipping; Hinge

Dystrophin is a member of the spectrin family of proteins, which are characterized by being predominantly composed of a number of tandem copies of the STR motif [1,2]. It is a relatively large protein, being ~427 kDa in size, which places it among the largest 0.5% of human proteins. The large number of copies of this STR motif – 24 in dystrophin – is largely responsible for this impressive size. These STRs are ~110 amino-acid motifs recognized as unique protein domains [6], and although there are no atomic-resolution structures for STRs of dystrophin, there are several structures available from other members of the spectrin family. In all cases, they are known from X-ray and NMR studies to form a triple α -helical bundles,

with the first and third helices parallel and the intervening second antiparallel — roughly forming a cylinder ~5 nm long and ~2 nm in diameter. These motifs are invariably found in multiple tandem copies, and this zigzag arrangement of the polypeptide backbone produces elongated rod shapes for the region as a whole. Thermodynamic interactions have been shown to exist between adjacent STR motifs in some cases [7–9], and these are thought to confer rigidity on the region as a whole so that multiple STRs merge structurally into one single long rod. This has been supported in some X-ray structures of some tandem STRs in which the third helix of one motif propagates directly into the first helix of the subsequent STR [10,11]. However, there are many STRs and the junctions between them vary — in some cases, intervening regions with no known homology are found, which are thought to disrupt these interactions, creating so-called hinge regions. In other cases, the relative helical propensity of amino acids at the STR termini next to certain uninterrupted junctions has been shown to affect the thermodynamic stability of the flanking STRs, possibly creating hinges of a different type [12].

The large size of dystrophin is problematic in many ways. Firstly, it provides a large cross section for random mutation, and thus explains DMD's high incidence and high genetic diversity. It is estimated that 30–50% of cases represent de novo mutations, and hundreds of distinct defects have been characterized [13,14]. Since this condition is common, fatal, and single factorial, it is an attractive target for gene therapy studies. Here too, the large size poses a problem, in that some leading viral gene delivery vectors cannot easily accommodate full length dystrophin. To facilitate delivery, edited minidystrophins have been studied, containing all requisite binding motifs, but having some of the STR regions removed [15–21]. Animal studies have been encouraging, so much so that human trials are poised to begin.

However, the large size and complexity does carry a silver lining: for many defects, the vast majority of the gene remains unperturbed. This has led to the development of the exon-skipping repair strategy for DMD, in which the damaged portion of the gene is skipped at the RNA level by using antisense oligonucleotide analogs to interfere with the spliceosome during processing of the primary transcript to the mature mRNA [22–24]. Since most mutations occur in the rod region — it forms the bulk of the gene, and contains both hotspots for mutation — this leads to rod-truncated dystrophin variants. However, the consequences on the rod region of these editing processes are unclear at present. Studies indicating significant inter-STR interactions suggest that effects may not be entirely local, and removal of one STR may affect other nearby remaining STRs. This may be behind context effects seen in the mini-dystrophin animal studies, in which similar constructs had different efficacies.

We may turn to nature itself for guidance in this matter. In fact, the one of the best minidystrophins is based upon a serendipitous discovery of a variant with exons 17–48 deleted from a patient with a very mild form of Becker Muscular Dystrophy [25] — an condition related to DMD in which an altered, less functional form of dystrophin is present and muscle deterioration greatly reduced. This deletion was chromosomal; however, there are other post-transcriptional deletions that occur with this gene even in normal people: several alternatively processed mRNAs are produced, in which one or more exons are skipped. These alternative transcripts are produced in a tissue specific manner, arguing for their biological relevance; and appear to be involved in the observed heterogeneity of clinical severity of certain BMD mutations [26,27]. However, these edits have been so far detected only in RNA, and it is unclear whether they result in functional protein — and whether the deletion has any effect on the properties of the modified dystrophin produced.

When examining these natural processing events in relation to the STR structure of dystrophin, some interesting patterns emerge. In general, each STR is coded for by two exons, with introns occurring either very nearly coincident with STR junctions, or at a point approximately 43%

(using the Koenig and Kunkel [5] alignment; 42% using the Winder [28] alignment) of the way through each STR. We have visualized this distribution in Fig. 1. The gene structure and STR boundaries are as given in Genbank accession NM004006, which corresponds to the Dp427m form. Intron locations were taken from the work of Roberts [4]. The intron locations relative to the STR structure were calculated by dividing the number of nucleotides into each STR that each intron occurs, the total number of bases in that specific STR. These values clustered, with introns alternately falling near an STR junction (i.e. near 0 or 1) or near the midpoint. We can classify these sites as end, e; or middle, m. Skipping of a single exon would be expected to result in a protein with a residual fractional STR. Such fractional domains have been considered to be unstable, and unlikely to be biologically relevant [29]. However, Sironi et al. [3] found that most in vivo events involved the skipping of an even number of exons, which would not leave such fractional motifs behind. However, in contrast to most engineered edits which have so far been constructed with deletions at STR boundaries, roughly corresponding to e–e events, natural multiple exon-skipping events are predominantly m–m. While m–m events do indeed remove an integral number of STRs, they do so by removing two halves, and leave behind two fractional yet potentially complimentary STR domains. When translated these regions have the potential to form a single hybrid STR motif as indicated schematically in Fig. 1. An unresolved question is whether or not such a hybrid structure is viable.

We hypothesized that some hybrid STRs, especially those predicted as products of naturally observed m–m fusions discussed above, could form viable structures. To test this, we produced a rod containing one such observed event [3] the deletion of exons 41 and 42, which code for regions at the of D15 and the beginning of D16 (Table 1). This RNA splicing event was observed to be brain specific, and so is potentially linked to the common side effect of mental retardation linked to DMD [30,31]. To explore this deletion, we started with a larger fragment, D14:17. Vector cDMD5b-7 [32] containing the D11 to D17 STR motifs was obtained from the ATCC (ATCC 57672), and used as a PCR template. Regions corresponding to D14:15, D16:17 and D14:17 were amplified by PCR with primers designed to hybridize to codons corresponding to appropriate amino acids as shown in Fig. 2. A four primer PCR protocol [33,34], with internal primers provided to skip sequences corresponding to exons 41 and 42 was used to produce the D14:17 Δ e41,42 variant. When producing recombinant fragments corresponding to specific STR regions of dystrophin, it is often not completely clear how to do it; the homology is relatively weak and there are no obvious delimiting features to indicate where to start and end. The original domain boundaries set out by Koenig and Kunkel [5] are often used, but alternative alignments are possible, for instance the Winder Gibson Kendrick–Jones alignment [28]. The maximum difference between these two alignments is 8 amino acids (although in the region we are concerned with much smaller differences of 1 to 3 are seen as shown in Fig. 2). However, to be conservative, a ~8 amino acid flanking region has been incorporated into our constructs so that no matter which alignment is more physically relevant, our constructs will encompass appropriate domains with minor flanking regions.

To facilitate cloning, the forward (5'-end; N-terminus of the protein) and reverse (3'-end, C-terminus) primers contained sequence to append a *Bam*HI restriction site (GGATCC); and an *Age*I/stop codon/*Eco*RI site (ACCGGTTTAAGAATTC). This appended a Gly–Ser dipeptide to the N-terminus of the fragment, and a Pro–Val to the C-terminus. These PCR products were cloned into a pGEX expression vector, which expressed the proteins as fusion peptides with glutathione–S-transferase. After affinity purification of the fusion protein, the dystrophin peptide was released by treatment with thrombin and purified by ion exchange chromatography as described. All vectors were sequenced and shown to be correctly constructed, and the proteins were analyzed by MALDI mass spectrometry, with experimental molecular masses being within 1% of the theoretical values. Proteins were substantially homogeneous by SDS-PAGE, as shown in Fig. 4.

Since STRs are highly α -helical, CD spectroscopy is a useful tool to monitor the proper folding of these recombinant fragments. Spectra were acquired using a Jasco J715 spectrometer in a 1-mm path length cell. For determination of molar ellipticity, samples with accurately determined $A_{280} \sim 0.7$ in PBS (5 mM Na phosphate pH 7.4, 150 mM NaCl) were used, and data acquired from 300 nm to 190 nm. All proteins showed substantially helical structure, as demonstrated by their CD spectra, Fig. 3. All peptides show the characteristic parallel and perpendicular helix transitions at ~ 208 and ~ 222 nm [35], although D14:15 was clearly less helical as we discuss below. Use of decomposition algorithms and basis sets derived from proteins of known structure can yield estimates of secondary structure of our proteins. For instance, least-squares fitting to a simple and robust three state basis set [36] gives an α -helical secondary structure percentages of 32%, 70%, 68% and 81% for D14:15, D16:17, D14:17 and D14:17 $_{\Delta e41,42}$, respectively. However, accuracy of these small basis set models is limited, and factors such as super-coiling of the three individual helices in the bundle can distort these spectra [37]. As such, in this family of proteins the θ_{222} value is often used as a proxy for helical content, with a value of -36000 corresponding to 100% helical structure [38]. This approach yielded similar results, since the spectra are very nearly dominated by the α -helical signal: 42%, 73%, 66% and 79%.

In any event, the high helicity suggests that the structures are well folded. X-ray structures of homologous motifs suggest that a well folded STRs will have $\sim 75\%$ α -helical content, with the rest of the residues found in turns connecting the three helices; D16:17 and D14:17 $_{\Delta e41,42}$ are well folded by this criteria. The only fragment that is problematic is in fact the unskipped D14:15, which was low, at 32 to 42% helical, or only about half of the expected value. D14:17 was similarly a bit low, presumably due to the D14:15 region contained in this larger fragment. The primary sequence homology is particularly weak in the D14–D15 regions, with substantial gaps and insertion found needed by both alignments. In fact, the D14 is missing both highly conserved W residues that have been shown to be important for proper folding [39], and D15 is missing the first one. As such, this low helicity value may be a manifestation of this poor homology, and is probably not an artifact of our recombinant expression system. What structure does form for D14:15 does seem to be quite stable as judged by its highly cooperative and high T_m thermal denaturation profile (Fig. 3B). In any event, we are more concerned with the nature of the hybrid motif, which seems to be quite well folded.

The globular nature of these proteins was also examined by looking for a cooperative transition during thermal unfolding. For thermal titrations the sample were set to have $A_{280} \sim 0.3$, and CD data acquired between 250 and 200 nm with the temperature scanned from 13 to 91 °C at 1 °C/min scanning using a Peltier cell holder. Fig. 3B shows that all peptides underwent a sharp, cooperative transition — even the less helical D14:15.

Data analysis was by standard procedures for these types of experiments [40]. Briefly, the ϕ_{222} was fit to a model in which the native and denatured states exhibit linear dependence of ϕ_{222} on the temperature, and a transition between these two lines occurs with an equilibrium determined by the free energy, ΔG , at the given temperature. For proteins, ΔG is typically represented by a constant heat capacity, C_p , model, formulated with the additional parameters written in terms of the transition enthalpy, ΔH_m , and temperature, T_m : $\Delta G = \Delta H_m(1 - T/T_m) + C_p(T - T_m - T \ln(T/T_m))$. Data were fit to this model by non-linear least squares fitting within the Origin software environment to yield estimates of T_m , ΔH and C_p . However, for multidomain proteins with transitions that are not well separated, ΔH is often underestimated as the two transitions blur into a single broad transition, which lowers the slope of the transition. As such, although apparent ΔH values are yielded, they are not meaningful without corroboration of the number of transitions involved. The T_m obtained, however, does tend to a true mean T_m , and so does provide a reliable measure of stability, and is reported. As seen in Fig. 3B, all proteins exhibited cooperative transition and had T_m s > 55 °C. The exon skipped

variant showed the lowest T_m the group at 55 °C; however it was still nearly completely folded at physiological temperature of 37 °C. In fact, many naturally occurring STR rods have lower T_m s, such as HE α 4:5, HE α 8:9, HE α 13:14, (STR fragments from human erythrocyte α -spectrin) [12], although others from that same work or other works [7] have higher T_m s. This hybrid STR rod in fact appears quite unremarkable with respect to thermal stability.

Finally, experiments were conducted to examine the domain structure of the proteins by assessing their resistance to non-specific proteolysis. Aliquots of protein containing 1.5 μ g in 10.0 μ l of PBS were prepared, except that the first aliquot had 2.2 μ g. A 4.62 μ l, 1.5 ng bolus of proteinase K was added to the first larger aliquot, it was rapidly mixed by pipeting several times, and this same 4.62 μ l volume was then removed and transferred to the second aliquot. By continuing this mixing and transfer, a semi-decadal dilution as indicated in Fig. 4 series was rapidly prepared. At the penultimate tube, the bolus was removed and discarded, while the last tube remained unadulterated as a control. The procedure was conducted in under 1 min; and the tubes were then incubated for 30 min at 37 °C, at which time they were transferred to 0 °C, loading buffer was added and the samples were heated to 99 °C for 5 min and run on SDS PAGE gels.

As seen in Fig. 4, only the D14:17 peptides exhibited any significant degradation intermediates. This peptide was rapidly proteolysed into fragments of approximately 2 STRs in size, consistent with cleavage between D15 and D16. This was corroborated by mass spectrometry analysis, which detected the emergence of several peaks at 25.7, 25.9 and 27.6 kDa. No further smaller intermediates were seen, suggesting that a 2 STR size constitutes a fundamental biophysical domain in this region. In contrast, the hybrid motif showed great resistance to proteolysis, with substantial persistence in the reaction, up to the highest protease concentration used (a mass ratio of 1:1500 substrate to protease), comparable to the stabilities of the recombinant 2 STR fragments D14:15 and D16:17. It is as yet not known whether the presence of this proteolysis sensitivity site (or its absence in a hypothetical, shorter exon-skipped rod) has any impact, positive or negative, on dystrophin's biological function. Interestingly, such sensitivity has long been correlated to with flexibility, in proteins in general [41] as well as in dystrophin [5]. However, this does show that edited rods are not necessarily simply slightly shortened versions of the native structure, but can be different in some qualitative way.

Taken together, the high helicity, cooperative unfolding transition, and resistance to proteolysis demonstrate that this exon skipped hybrid STR can form a stable structure and be part of a viable protein. In fact, increased resistance to proteolysis compared to the native structure suggests that such variants in intact dystrophin may very well play different biological roles. Sensitivity to proteolysis correlates with flexible or disordered structure, and the site detected between D15 and D16 corresponds to a region previously identified as containing a short, non-STR intervening peptide [5]. While it is shorter than the other such regions that have come to be identified as the four hinges of dystrophin, there is no homology or even definite size for these regions, so it is difficult to objectively exclude this region. This high sensitivity suggests that this region between D15 and D16 might also be deserving of hinge status.

The deletion of exons 41 and 42 removes a region spanning this intervening region, and so it is not surprising that the hybrid rod so produced would be more refractory to proteolysis. What was not clear at the outset (at least to the author) is whether a hybrid STR structure would be stable. While domain shuffling – the movement of intact domains within a protein family or even across families – is well documented and appears to be the rule not the exception [42, 43], the splicing of fractional domains to produce viable hybrid domains is much less well known. In some studies, exon-skipping events that disrupt the hydrophobic core of domains have been considered to be non-viable [29]; we show here that this is not necessarily the case.

It is interesting to speculate about the purpose of these exon splicing events. The exon 41–42 event studied here is brain specific [3], and does not occur in muscle. Mental retardation is a significant auxiliary symptom observed in some but not all cases of DMD, and it may be that this alternatively spliced RNA variant produces an isoform of dystrophin essential for proper neurological development or function, and DMD mutations that interfere with this splicing event may disrupt this auxiliary function. Dystrophin is also involved in X-linked cardiomyopathy, (which often also arises in DMD and BMD patients), and some other exon-skipping events are found in specifically cardiac muscle.

These exon-skipping events may be of particular interest to BMD, which often is a result of in-frame exon deletion events, which allows translation to continue to the normal C-terminus of the protein, albeit with some intervening sequence removed. It has also been shown that the specific pattern of exon skipping, even among the remaining exons, varies in a complex manner among patients with very similar underlying deletions, but with varying clinical severity.

Interestingly, the most common type of all the multiple exon splicing events observed is m–m. A minor e–e population is also seen, but heterologous e–m or m–e event are strongly under-represented. This suggests that hybrid STR production is the rule and not the exception *in vivo*. In contrast, most man-made edited rods – for instance for minidystrophin gene therapy – have been constructed by deletions placed at STR-STR junctions, roughly corresponding to e–e events. In some cases, these modified dystrophins were less than optimally effective. It may be that deletions more in accord with naturally occurring events may be more well tolerated and more effective.

Exon-skipping therapy may also be informed by this conceptualization. It appears that while skipping of a single damaged exon, while restoring the reading frame and allowing production of near full-length dystrophin, may also carry consequences to physical properties of the rod that would be problematic. During the course of this work, we attempted to produce single exon skipped D14:17 Δ e41 and D14:17 Δ e42; these efforts were unsuccessful and only degraded fusion protein was produced in our recombinant system. While we cannot be certain since we were not able to obtain these proteins for study, this suggests that these fractional-STR containing rods are unstable. It has been recognized that skipping of two or more exons may be better than skipping a single exon [44], and this work provides justification for examining a wider set of m–m bi-skipped events than may have previously been considered. Clearly, a full examination of all the *in vivo* observed events to determine their consequences on the dystrophin rod will provide much useful information and may shed light on alternative functions of dystrophin in the brain, heart and elsewhere. In fact, both exon skipping and minidystrophin gene replacement clinical trials are in their early stages, and this information will become increasingly useful and needed.

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Fig 1.

Hybrid STR production. Top: the location of each intron insertion sites relative to the STR boundaries is shown. These boundaries cluster into two regions, falling either near the middle, m (~43% of the way through), or near the edges, e, of each STR. The numbers associated with the m points indicate which STR the intron lies in. Bottom: a schematic illustrating how exon-skipping events can produce hybrid STR motifs. The primary transcript is indicated at the top, with rectangles indicating the exons, and introns being the thin line. STR motifs are represented by the S-shaped structures; two exons code for each STR, and they are linked by identical shading. Exon skipping by removal of pairs of exons at m junctions would produce a hybrid STR motif made up of two complimentary portions of the remaining STRs.

Fig 2.

Construction of the recombinant fragments used in this study. Fragments were based upon a parent D14:17 fragment of dystrophin, roughly encompassing exons 38 through 45. The amino acid sequence at each STR motif boundary regions are shown under each expansion triangle; the exact junctions as indicated by the Koenig and Kunkel, KK, or Winder Gibson Kendrick-Jones, WGK, alignments are indicated. To account for any disagreement – KK and WGK differ by up to 8 amino acids in other regions – a safety margin of 8 amino acids from the KK alignment was used to begin and end our constructs, which are indicated by the lines with the thick arrows. The hybrid motif was constructed by deleting the exon 41 and 42 regions; the amino acids corresponding to these junctions are shown.

Fig 3.

Spectroscopic properties. (A) All peptides showed substantially α -helical structures, with D14:15 the least helical and the hybrid D14:17_{Δe41,42} the most. (B) All peptides appeared to undergo a cooperative thermally induced folding transition. In this case, the hybrid D14:17_{Δe41,42} was the least stable with a T_m of 55 °C. The wild type peptides D14:15, D16:17 and D14:17 exhibited T_m s of 67, 69 and 58 °C respectively. Although the hybrid motif showed lowered relative stability, it still appears to be substantially folded at physiological temperatures, displays a cooperative transition, and was the most helical of all the peptides.

Fig 4.

Resistance to proteolysis. Serial dilution of a non-specific protease, Proteinase K, was used to assess the domain structure and stability of these proteins. A constant amount of sample proteins, $\sim 1.5 \mu\text{g}$, was incubated with 0 to 1 ng of protease as indicated. D14:17 is digested to fragments consistent with cleavage between D15 and D16, while the hybrid motif D14:17 Δ e41,42 is comparatively more stable, resisting digestion nearly as well as the 2-STR D14:15 or D16:17 motifs. The center lane contains molecular mass markers (14, 25, 28, 39 kDa).

Table 1

Proteins studied

Protein	Exons skipped	Hybrid STR?	$\theta_{222}(\text{kdeg cm}^2/\text{dmol})$	Protease sensitive?	T_m (C)
D14:17	none	No	-23.6	Yes	58.1
D14:15	none	No	-15.5	No	67.4
D16:17	none	No	-26.4	No	68.6
D14(15" 16)17	41,42	Yes: (15" 16)	-28.1	No	54.6

The most significant properties of the proteins studied are summarized.