Mechanistic and functional diversity in the mechanosensory kinases of the titin-like family

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Abstract

The giant cytoskeletal kinases of the titin-like family are emerging as key mediators of stretch-sensing in muscle. It is thought that their elastic conformational deformation during muscle function regulates both their catalysis and the recruitment of regulatory proteins to signalosomes that assemble in their vicinity. In the present article, we discuss the speciation of mechanosensory mechanisms in titin-like kinases, their scaffolding properties and the kinase/pseudokinase domain variations that define a rich functional diversity across the family.

Titin-like kinases

The cytoskeleton of muscle cells contains giant filamentous proteins of the titin-like family (0.7–4 MDa) that mediate the sensing and transduction of mechanical signals in the myofibril. Such mechanical signals drive the development and regulation of the tissue in adaptation to physical demands. Titin-like proteins are composed of numerous Ig-like domains linked in series and contain one or two kinase domains invariably located near their C-termini. Members of this family include titin and obscurin in mammals; twitchin/UNC-22, the obscurin homologue UNC-89 and the small titin TTN-1 in nematodes; twitchin in molluscs; and projectin, UNC-89 and stretchin in insects [1–3]. Despite acute variations in the length and domain organization of these proteins, their kinase regions are highly conserved, consisting of Fn (fibronectin)–linker–kinase–tail–Ig domains (Figure 1). This evolutionary conservation led researchers to anticipate a shared regulatory mechanism of the kinase domain and related signalling roles in muscle. However, recent findings point to a rich mechanistic and functional diversity among kinases of this family. In the present article, we review the currently emerging concepts regarding titin-like kinases.

Intrasteric regulation by kinase-flanking extensions

The crystal structure of the Fn–linker–kinase–tail–Ig region of *Caenorhabditis elegans* twitchin (TwcKR) has been elucidated recently [4]. This shows the kinase domain within its molecular context, revealing the connections that anchor

and orient it in the filament: the kinase is wrapped by both N- and C-terminal flanking tails that pack tightly against it, with Fn and Ig domains tethering this kinase assembly (Figure 1b). The CRD (C-terminal regulatory domain) tail wedges itself between the two kinase lobes, blocking the binding sites for ATP and the phosphorylatable protein substrate. This CRD conformation reproduces that observed in previous structures of TwcKs (twitchin kinases) from *C. elegans* [5,6] and *Aplysia* [6] and human TK (titin kinase) [7]. Conversely, the TwcKR structure has revealed for the first time that the NL (N-terminal tail) is not an unstructured segment as previously assumed. Instead, it forms a ‘crown’ that rests on the back of the interlobular kinase hinge region, with its remaining chain folded against the N-terminal kinase lobe, sprawling across the $\beta 1$ – $\beta 2$ hairpin (containing the glycine-rich loop in the ATP-binding pocket) and leading into a frontal β -hairpin loop that connects the kinase assembly to the preceding Fn domain. The NL crown packs against the kinase through a conserved aromatic motif (YXXYXFFXXWXXYY) (Figure 1b), but does not occupy the canonical hydrophobic pocket of AGC kinases [8]. The NL also establishes direct contacts with the catalytic helix αC . These multiple interactions with helix αC , $\beta 1$ – $\beta 2$ hairpin and kinase hinge indicate that this extension is also a regulator of TwcK catalysis.

Kinetic data for TwcKR variants confirmed that the bare kinase domain has maximum phosphotransfer activity, whereas both NL and CRD extensions are inhibitory [4]. Separately, each segment approximately halves phosphotransfer levels, but jointly they efficiently silence catalysis. The inhibition provided by the NL segment appears to be due to a restriction of intramolecular hinge motions required for catalysis (characterized in other protein kinases [9,10]). In contrast, CRD-mediated inhibition is caused by the blockage of the active site (Figure 1b). It is remarkable that high levels of catalysis are observed in the presence of the CRD. However, the CRD segment that directly blocks the active site folds into a 3_{10} helix, a rare chain conformation that is

Key words: intrasteric regulation, mechanosensing, obscurin, (pseudo)kinase scaffold, titin-like kinase, twitchin.

Abbreviations used: AFM, atomic force microscopy; CPNA-1, copine domain protein atypical 1; CRD, C-terminal regulatory domain; CTD, C-terminal domain; Fn, fibronectin; MD, molecular dynamics; MuRF, muscle-specific RING finger; NL, N-terminal tail; SCPL-1, small CTD phosphatase-like 1; TK, titin kinase; TwcK, twitchin kinase; TwcKR, twitchin kinase region.

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Figure 1 | The conserved kinase region of titin-like proteins

(a) Schematic domain composition. (b) Crystal structure of the TwcKR from *C. elegans*. Lateral (left) and frontal views (right; flanking Fn/Ig domains omitted to improve visibility) illustrating the packing of NL and CRD extensions against the kinase catalytic core. The interaction of the NL with the kinase hinge region (through aromatic groups), the $\beta 1$ - $\beta 2$ hairpin (blue) containing the glycine-rich loop (G), and the helix αC (broken circle) is shown. The CRD- $\alpha R2$ helix blocking the ATP-binding site is indicated by an arrowhead. Other catalytically important groups are shown and labelled as follows: catalytic aspartate (D), ATP-co-ordinating lysine (K) and DFG motif.

energetically unfavourable. It is conceivable that this segment might be out-competed by the substrates in the absence of the NL, possibly through a local unfolding–folding transition. In any case, activation of the NL–kinase–CRD assembly must require tail removal to some extent.

Mechanoactivation

The mechanism of tail release in titin-like kinases remains controversial. To date, protein activators that bind and displace these tails from the kinase active site have not been identified. However, it has recently been hypothesized that kinase activation is a direct result of the mechanical deformation of the cytoskeleton during myofibril stretch [11,12]. This hypothesis was based on AFM (atomic force microscopy) data [12–14] and force-probe MD (molecular dynamics) simulations [11] on human TK that indicated that stretch pulls the CRD from the kinase core, unfolding it and freeing the active site. It was then concluded that TK functions as a biological stress sensor, which is in agreement with the observed functional association of TK with stretch-activated pathways in the myofibril [15].

This mechanism of activation, however, does not seem to be shared by all members of the family. AFM investigations of twitchin and TTN1 (titin 1) kinases from *C. elegans* failed to detect the distinct unfolding of their CRD tails [16]. Steered MD simulations on TwcKR [4] then indicated that the NL in this kinase is mechanically labile, but that the

CRD is poorly responsive to stretch and acts as an integral structural component of the C-terminal kinase lobe, to which it remains bound. This, together with the observed catalytic tolerance of this tail in TwcK, points to the possibility that the CRD might maintain extensive stabilizing contacts with the kinase domain throughout the catalytic cycle, protecting the active site against mechanical damage. In fact, stretching of the exposed kinase domain could be expected to hinder the interlobe closure motions required for catalysis, resulting in inhibition. Stretch-inhibition has been demonstrated in enzymes such as guanylate kinase, where the attachment of a DNA spring that introduced tension and halted interdomain closure also hindered catalysis [17]. In TwcK, the CRD might enable catalysis under tension by aiding intramolecular reorganization and providing fold stability.

The contrasting data on human TK and nematode TwcK correlates with actual differences in the composition, structure and interactions of their CRD tails. The CRD of TwcK is longer and more structured than that of TK and thus mechanically more resistant. This finding suggests that activation through CRD stretch-unfolding is not a generic mechanism of titin-like kinases, but that at least two mechanosensing modes exist in the family, leading to ‘priming’ or ‘baring’ of the active site. In ‘priming’, stretch might primarily release the NL barrier, whose removal might then permit catalysis in the presence of the CRD stabilizer. The ‘baring’ mechanism, on the other hand, might primarily

Figure 2 | Scaffolding interactions of titin-like kinases

Protein interactions mediated by (a) human TK and (b) UNC-89 kinases from *C. elegans*. Domain colour code for TK and UNC-89 as in Figure 1. Details on interactions are given in the text. ILK, integrin-linked kinase; SRF, serum-response factor.

emphasize the exposure of the kinase surface, possibly also involving the release of the CRD tail as observed in TK. These two possible modes of mechanosensing might differentially affect the distinct catalytic and scaffolding roles of the kinase domains in this family.

Titin-like kinases as protein scaffolds

Recently, a number of interacting partners have been identified for titin-like kinases, indicating that scaffolding plays an important part in their signalling. Human TK interacts with the autophagosomal receptors Nbr1 and p62, and the E3 ubiquitin ligase MuRF2 (muscle-specific RING finger 2) [15] (Figure 2a). The formation of this signalosome of turnover factors is transient and appears to be dictated by the stretch-induced conformation of TK's CRD. Upon mechanical arrest, the release of MuRF2 leads to the repression of the transcriptional response of serum-

response factor, linking mechanosensing with gene expression regulation in striated muscle. In addition, the conserved Ig-Fn tandem immediately preceding TK interacts with the ubiquitin ligase MuRF1 [18,19] that is linked to muscle atrophy [20,21]. Taken together, the data point to TK as an important mechanosensory signalling node in the cytoskeleton of vertebrate muscle. Notably, no binding partners are known for twitchin, TTN-1 or projectin kinases in invertebrates (the closest TK homologues) and MuRF homologues have also not been identified in that animal group. Thus, to some extent, signalling through titin-like kinases might be phylum-specific.

The kinases within the obscurin filament system (UNC-89 in invertebrates) also regulate protein turnover in muscle. Obscurin/UNC-89 contain two kinase domains, PK1 and PK2, linked by a sequence of 450–1100 residues depending on the organism [22]. This interkinase linking region consistently has Ig-Fn domains N-terminal to PK2, but otherwise is

a low-complexity sequence with high proline content and no evidence of shared homology [22]. These features are reminiscent of the well-known molecular springs of titin (PEVK, N2B and N2A [3]), suggesting that the interkinase linker is an elastic component of the obscurin/UNC-89 mechanosensory kinase node. In *C. elegans* UNC-89, the Ig-Fn domains preceding PK2 interact with MEL-26, a substrate-recognition protein for cullin-3 [23] (Figure 2b). Cullins are scaffolds for the assembly of the ubiquitin degradation machinery, including E3 ubiquitin ligases [24]. In early *C. elegans* embryos, a target of the cullin-3–MEL-26 complex is the microtubule-severing enzyme katanin (MEI-1) [25]. The loss- or gain-of-function of either *mel-26* or *mei-1* results in disorganization of thick filaments similar to *unc-89* mutants [23]. It is thought that the interaction of UNC-89 with MEL-26 inhibits the activity of the cullin-3–MEL-26 complex in promoting the ubiquitin-mediated degradation of MEI-1, whose activity is required for myosin thick filament organization. Similarly, studies on an obscurin-knockout mouse revealed that degradation of sAnk1.5 (important for sarcoplasmic reticulum architecture in striated muscle) is dependent upon obscurin and a cullin-3 substrate-recognition protein, KCTD6 (K⁺ channel tetramerization domain-containing 6) [26]. These studies point to an evolutionarily related mechanism in invertebrate/vertebrate muscle by which UNC-89/obscurin regulates ubiquitin-mediated protein degradation.

In addition to those interactions, each kinase domain in *C. elegans* UNC-89 interacts with SCPL-1 [small CTD (C-terminal domain) phosphatase-like 1], a CTD-type protein phosphatase [27] (Figure 2b). CTD phosphatases are known to regulate transcription [28,29], but this finding suggested a new function for this class of proteins in muscle-specific signalling. In agreement, *scpl-1* mutants had defective egg-laying muscle function [27]. Furthermore, both PK1 and the interkinase region interact with LIM-9 [30], the nematode homologue of the human protein FHL (four-and-a-half LIM domains) that in vertebrate muscle associates with the elastic I-band of titin [3]. UNC-89, LIM-9 and SCPL-1 interact with CPNA-1 (copine domain protein atypical 1), a copine domain protein that localizes to integrin-adhesion sites (M-lines and dense bodies) in the body-wall muscle of *C. elegans* [31]. CPNA-1 binds to the M-line/dense body protein PAT-6 (actopaxin/ α -parvin) [31], which in turn interacts with PAT-4 (integrin-linked kinase) associated with integrins [32]. Nematodes lacking CPNA-1 are embryonic lethal and display disruption of the myofilament lattice, with UNC-89 becoming misplaced into large foci [31]. Thus CPNA-1 functionally links UNC-89 kinases with integrins, thereby connecting main cytoskeletal and cell-surface sensory pathways (Figure 2b). It remains to be discovered whether this functional coupling is also present in vertebrate obscurins.

These data suggest that, through their scaffolding, titin-like kinases might act as cross-talk nodes that integrate cytoskeletal mechanics with general regulatory pathways in the myofibril. However, it must be considered that

these kinases are non-diffusible, so their function must be strongly conditioned by their location in the myofibril. The importance of this observation is highlighted by the latest data on mouse obscurin kinases. It was found that a small extracellular isoform of obscurin exists at the muscle cell surface where PK2 associates with the extracellular β 1 subunit of Na⁺/K⁺-ATPase [33]. Although the physiological significance of this finding is unknown, it illustrates how compartmentalization might result in multiple and unsuspected functions for these kinases.

Evolutionary dichotomy of kinases and pseudokinases in the titin-like family

The catalysis of titin-like kinases remains poorly characterized. Physiological substrates are unidentified for most members of the family and several titin-like kinases are atypical, including the TK representative [34]; only twitchin and TTN-1 kinases have been shown to exhibit high levels of catalysis *in vitro* [4,35–37]. However, the most prominent example of catalytic divergence in this family is that of obscurin kinases that, despite high levels of sequence identity, have segregated into kinases and pseudokinases according to animal phyla. In vertebrate obscurin, PK1 and PK2 have canonical active motifs (Figure 3) and have been shown experimentally to undergo autophosphorylation [33]. In contrast, invertebrate UNC-89 kinases have atypical active sites and some might be inactive (Figure 3). UNC-89-PK1 from insects has classical ATP-binding motifs, but has lost the typical HXDXXKXXN motif in the catalytic loop [38]. This has become HXGXTXXD, where the catalytic aspartate residue is replaced by an unreactive glycine residue and the frequently occurring lysine and asparagine groups assisting ATP co-ordination and phosphotransfer are replaced by threonine and aspartate. The new motif is highly conserved across all insects, pointing to the evolution of a new function or specificity of PK1 in that animal group. In contrast, in nematode UNC-89-PK1, the catalytic motif is present, but ATP-binding residues are degenerated, including the conserved lysine residue in strand- β 3 that is now a glutamine residue. This exchange might influence catalysis or specificity. Interestingly, this observation adds to the existence of *C. elegans* UNC-89 isoforms -C and -D that have truncated non-functional PK1 domains lacking the N-terminal lobe [22]. Thus PK1 domains in insects and nematodes have potentially undergone a convergent evolution towards being catalytically altered pseudokinases, which suggests that this feature is crucial to function in invertebrate muscle. In contrast, UNC-89-PK2 has typical active motifs, apart from a somewhat modified glycine-rich loop and the fact that the catalytic aspartate residue is asparagine in several organisms (glutamate in one other member) (Figure 3 and Supplementary Figure S3 at <http://www.biochemsoctrans.org/bst/041/bst0411066add.htm>). Mutagenesis studies in model protein kinases revealed that such asparagine/glutamate mutations are strongly inactivating [39–41]. However, the atypical kinase JAK2 (Janus kinase 2) contains an

Figure 3 | Catalytic motifs in kinase and pseudokinase domains of obscurin/UNC-89
The roles of the residues in these sequence motifs are described in [38]. Explicit sequence alignments are provided as Supplementary Figure S1 at <http://www.biochemsoctrans.org/bst/041/bst0411066add.htm>.

PK1					PK2				
		G-rich loop (GxGxxG)	ATP-binding motif (AxK)	Catalytic motif	DFG motif	G-rich loop (GxGxxG)	ATP-binding motif (AxK)	Catalytic motif	DFG motif
(n = 21)	Human/ Primate	GxGxxG	AxK	HxDxKxxN	DFG	[Q/R]xGxxS	AxK	HxDxKxxN	DLG
	Other mammals	Gx[S/A]GxxG				RxGxxS	AxK		D[L/F]G
	Bird	GxGxxS				KxGxxS	AxK		DFG
	Fish	GxGxx[G/S]				[N/A]xGxx[G/S]	[A/L]xK		D[L/F]G
(n = 26)	Drosophila	GxGxxG	AxK	HxGxTxxD	DFG	AxGxxS	VxK	HxNxKxxN	DFG
	Mosquito		AxK	HxGxTxxD		SxGxxS	VxK	HxDxKxxN	DFG
	Ant		AxK	HxGxTxxD		SxGxxS	[V/I]xK	Hx[D/N]xKxxN	DMG
	Beetle		AxK	HxGxTxxD		[S/A]xGxxS	[A/V]xK	Hx[D/N]xKxxN	D[F/M/L]G
	Louse		AxK	Hx[G/S]xTxxD		AxGxxS	VxK	Hx[D/N]xKxxN	DFG
	Butterfly		AxK	HxGxTxxE		WxGxxS	VxK	Hx[D/N]xKxxN	DFG
	Bee		AxK	HxGxTxxD		[Y/A]xGxxS	VxK	HxDxKxxN	DMG
(n = 4)	Nematodes	ExAxxG	LxQ	HxDxRxx[T/A/V]	DFG	[F/Y/P]xGxx[G/S]	[A/M]xK	Hx[D/N]xKxxN	DF[G/A]

asparagine residue at this location while maintaining activity, indicating that this exchange can be compensated for within the kinase machinery [42]. Hence it can be concluded that the titin-like kinase family comprises kinases, atypical kinases and barren pseudokinases with widely ranging catalytic properties. It has yet to be established which members of the family act as true mechanotransducers by mediating stretch-activated phosphotransfer and which might exploit mechanosensing primarily through other functions, e.g. conformationally regulated scaffolding.

Conclusion

Titin-like kinases form mechanosensory signalling nodes central to myofibril regulation. The speciation of their elastic regulatory segments coupled to kinase/pseudokinase catalytic variations, cellular compartmentalization and the establishment of distinct scaffolding interactions provides specificity in their sensing, supporting a range of specific muscle responses to stress stimuli.

Funding

We acknowledge the financial support of the Human Frontiers Science Programme [grant number RGP0044/2012].

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