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The Anatomy of a Molecular Giant: How the Sarcomere Cytoskeleton is Assembled from Immunoglobulin Superfamily Molecules

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D. O. FÜRST AND M. GAUTEL. The Anatomy of a Molecular Giant: How the Sarcomere Cytoskeleton is Assembled from Immunoglobulin Superfamily Molecules. *Journal of Molecular and Cellular Cardiology* (1995) 27, 951–959. Cross-striated muscle contains an elastic cytoskeleton comprised of the giant protein titin and several associated proteins. cDNA sequencing revealed that all these proteins are immunoglobulin superfamily members. This modular structure opens the possibility to dissect the proteins involved into functional units and to approach the problem of structure–function correlation at the molecular level.

KEY WORDS: Titin; C-protein; M-protein; H-protein; Myomesin; Twitchin; Immunoglobulin superfamily; Fibronectin repeat; Immunoglobulin domain; Myogenesis.

Introduction

Cross-striated muscle evolved with higher animals in order to fulfil their demand for intentional and coordinated movements, which have to be performed at greater precision and speed. The generally accepted "two-filament-sarcomere model" (reviewed e.g. by Huxley, 1990) could not, however, explain important properties of the muscle cell: upon passive stretch, for instance, muscles develop resting tension. Therefore, several attempts were made to extend this model to explain resting tension and intrinsic elasticity by postulating the presence of a third, elastic kind of filamentous system. The efforts to define such a third filament type were of limited success and lead to conflicting views of the nature of these structures (see review by Wang, 1985). A comparison of intact and skinned single muscle fibres clearly showed that at least up to a sarcomere length of 3.8 μm—contrary to the general belief—resting tension cannot be attributed to connective tissue but instead arises in the elastic resistance of the myofibril itself (Magid and Law, 1985). Likewise, the striking regularity of thin and thick filament geometries cannot be explained by the intrinsic self-assembly properties of their major constituent proteins, actin and myosin. These can assemble into filamentous structures *in vitro* that bear many morphological and functional features of native filaments; their precisely tailored lengths, however, are usually not observed *in vitro*. Surprisingly, thick filaments could be reconstituted in myosin-extracted sarcomeres in a way that rendered them virtually indistinguishable from native filaments (Maw and Rowe, 1986). Obviously important factors that control the precise assembly

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of sarcomeres are therefore usually lost during the fractionation of myofibrillar components.

New sarcomeric proteins are discovered

The discovery and subsequent characterization of new proteins in the myofibril raised broader attention for this subject. Low porosity gels had allowed for the first time the detection of two proteins in the megadalton range (Maruyama *et al.*, 1977; Wang *et al.*, 1979). The larger one, termed titin (or connectin), migrates as a doublet and is found only in sarcomeric muscles of vertebrates; the second protein, called nebulin, is absent from heart muscle and is therefore considered to be less important for the basic structure of the cross-striated myofibril (Wang and Williamson, 1980). Important support for the fundamental significance of an elastic structure whose main component could be titin came from experiments where selective degradation of titin by ionizing radiation greatly reduced the ability of skinned muscle fibres to generate passive tension (Horowits *et al.*, 1986). At the same time, thick filaments became misaligned. Thus, a dual role for titin was proposed: on the one hand, it should provide axial continuity between the two filament systems in order to be able to produce resting tension; on the other hand titin should keep thick filaments in register in the centre of the sarcomere, thus enabling the directed sliding of thick and thin filaments upon contraction (Horowits and Podolsy, 1987). This proposed role for titin necessarily constrains its localization. Nevertheless, early efforts using polyclonal antibodies resulted in conflicting views of the exact disposition of titin in the sarcomere (Maruyama *et al.*, 1985; Wang, 1984). A panel of monoclonal antibodies directed against a series of distinct epitopes along the protein demonstrated that single titin molecules extend all the way from the Z line through the half sarcomere up to the M band (Fürst *et al.*, 1988; Fürst *et al.*, 1989b). These as well as other studies also revealed that titin is firmly anchored along the thick filament, while it can be stretched at least four-fold over its I band portion (Fürst *et al.*, 1988; Itoh *et al.*, 1988; Whiting *et al.*, 1989; Wang *et al.*, 1993). The impressive molecular dimensions of titin were also demonstrated by electron microscopy of the purified protein (Maruyama *et al.*, 1984; Trinick *et al.*, 1984; Wang *et al.*, 1984). The most conclusive demonstration was from Nave *et al.* (1989): these pictures showed a long and thin rod (approximately 1 μm in length, 3–4 nm diameter) bearing a conspicuous head structure at one end. This head reflects the M band-anchoring portion of titin and includes two M line proteins (Nave *et al.*, 1989; see also below). An exhaustive panel of biochemical and biophysical methods was used to address crucial questions about titin, like for instance its mass, length, intrinsic elasticity and shape (Maruyama *et al.*, 1986; Hainfeld *et al.*, 1988; Kurzban and Wang, 1988; Nave *et al.*, 1991; Nave *et al.*, 1989). The results consistently indicated a long, flexible protein of a molecular mass in the range of 2.5–3 million that exhibits largely β-sheet structure. However, such methods, using the whole 1 μm polypeptide, are at a loss to explain in molecular detail the mechanisms of elasticity (Soteriou *et al.*, 1993a; King, 1994) or to map the ligand interactions of the molecule in a spatially resolved manner. In order to approach these questions at the molecular level it is necessary to gain access to the complete primary structure of the 3 MDa protein.

How titin is assembled from modules

Over the last years, the advances in large-scale sequencing technology have made the primary structures of a family of related proteins available. The first molecule whose complete primary structure became available was the mutationally defined *unc*-22 gene product from the nematode *Caenorhabditis elegans* (Moerman *et al.*, 1988; see below). Twitchin was found to be an unusually large modular protein (molecular mass ~750 kDa) mainly consisting of regularly arranged 100 amino acid domains that belong to the immunoglobulin superfamily (Benian *et al.*, 1989). This general principle of a modular architecture is shared by other invertebrate giant muscle proteins (Ayme-Southgate *et al.*, 1991; Fyrberg *et al.*, 1992; Lakey *et al.*, 1993) as well as vertebrate titin. The complete primary structure of titin is expected to be available in the near future (Labeit *et al.*, in preparation). The analysis of titin begun with cDNA contigs isolated from human cDNA expression libraries using A band titin-specific antibodies. These cDNAs showed a repetitive pattern of two kinds of modules that belong to the immunoglobulin superfamily: fibronectin class III (titin type I domains) and immunoglobulin C2 (titin type II domains) repeats (Labeit *et al.*, 1990). Extensions of these sequences soon revealed a more complex picture: in the C-region of the A band (Sjöström and Squire, 1977) these type I and II modules are arranged in super-repeats of 11 modules each (Labeit *et al.*, 1992). As the predicted size of one module is about 4 nm (Holden *et al.*, 1992), the expected extent of one

11 domain super-repeat is 44 nm. This closely reflects the spacing of the 11 transverse stipes observed in electron micrographs (Sjöström and Squire, 1977). Analysis of the domains within several super-repeats of titin revealed a higher degree of similarity between domains 11 positions apart, even in sequence stretches that are not crucially involved in the folding of Ig domains (Labeit *et al.*, 1992; Higgins *et al.*, 1994; Politou *et al.*, 1994a). Originally the concept of a molecular ruler was proposed for titin's role to tailor thick filaments to a uniform length (Whiting *et al.*, 1989; Trinick, 1992). Although this hypothesis awaits experimental evidence, the finding of the 11 domain super-repeat in the C-region suggests that this concept may well hold true for interactions with accessory proteins (see below). A number of further cDNAs has confirmed a high degree of inter-species homology of A band titin (Labeit *et al.* 1992; Fritz *et al.*, 1993) up to the A/I band junction (Tan *et al.*, 1993), as might be expected from a molecule involved in multiple protein–protein interactions.

The linking of cDNA contigs, derived from independent clones and obtained by antibodies mapped ultrastructurally, also allowed the orientation of titin in the sarcomere to be defined. Thus the N-terminus was placed in the Z line and the C-terminus in the M band (Labeit *et al.*, 1992).

Towards the M band a catalytic protein kinase domain, similar to the myosin light chain kinase family, was found. The presence of a kinase domain now seems to be the common denominator of the "giant muscle protein superfamily", which includes titin, twitchin, projectin and the molluscan minititins, as revealed by DNA sequencing and immunological methods (Benian *et al.*, 1989; Nave *et al.*, 1991; Labeit *et al.*, 1992; Vibert *et al.*, 1993; Heierhorst *et al.*, 1994). This group of muscle proteins may therefore also be regarded as a family of unusually large protein kinases of yet unknown function. The conservation of the molecular organization of the P-region modules (Labeit *et al.*, 1992) and of the kinase domain from nematode to man, argues for a crucial function in muscle. The M band region of titin (located C-terminally to the kinase domain) consists of a complex array of type II modules, interspersed by unique sequence insertions with no homology to other known proteins. Within one of these insertions, four copies of the amino acids VKSP are repeated in analogy to the multi-phosphorylation repeat of neurofilaments (Gautel *et al.*, 1993b). A related RSP motif was reported from an avian cDNA reactive to the Pc 1200 antibody (Maruyama *et al.*, 1994), however, the position of this antibody is not unambiguously defined.

The module patterns of I band titin seem to be of a completely different organization if compared to the super-repeat arrangement of the A band. A cDNA derived from the main immunogenic region of human cardiac titin near the A/I junction showed both type I and type II domains in an irregular pattern (Gautel *et al.*, 1993a). Partial cDNA sequences from embryonic avian I band titin suggest that immunoglobulin C2 modules may be an abundant building block along this portion of titin (Maruyama *et al.*, 1993). Since this region of titin is also the part that is extensible upon sarcomere stretch and is likely to show elastic behavior, the study of this molecular region should, in the long-term, enable us to understand the molecular basis of titin elasticity.

The making of modules

The most universally used module of cardiac titin is the immunoglobulin C2 domain (Labeit *et al.*, in preparation). The sequence similarity of these modules to other members of the immunoglobulin superfamily (see below) is reflected in an analogous fold of the amino acid chain, resulting in a beta barrel with seven strands that tightly buries the invariant tryptophane (Holden *et al.*, 1992; Politou *et al.*, 1994a). This common fold displays remarkable, though variant, stability in all regions of the sarcomere (Politou *et al.*, 1994a, b). Although intrinsic extensibility of A band titin has been demonstrated (Higuchi *et al.*, 1992), there is no indication that A band titin extends significantly at physiological salt conditions or sarcomere lengths (see above). Differences in melting temperatures and urea denaturation midpoints for modules from this region are therefore more likely to reflect "neutral" changes or sequence differences necessary to immobilize different ligands rather than to play a mechanical role. In addition, little is known about stabilizing interactions that may occur between neighbouring modules and differences in stability could well be due to a loss of such neighbouring domains or seemingly "useless" linkers (Politou *et al.*, 1994a). This highlights the general problem of dissecting large modular proteins into functional units.

Interactions with other sarcomeric proteins

If indeed the main task of titin is to stabilize the contractile apparatus and to maintain sarcomere

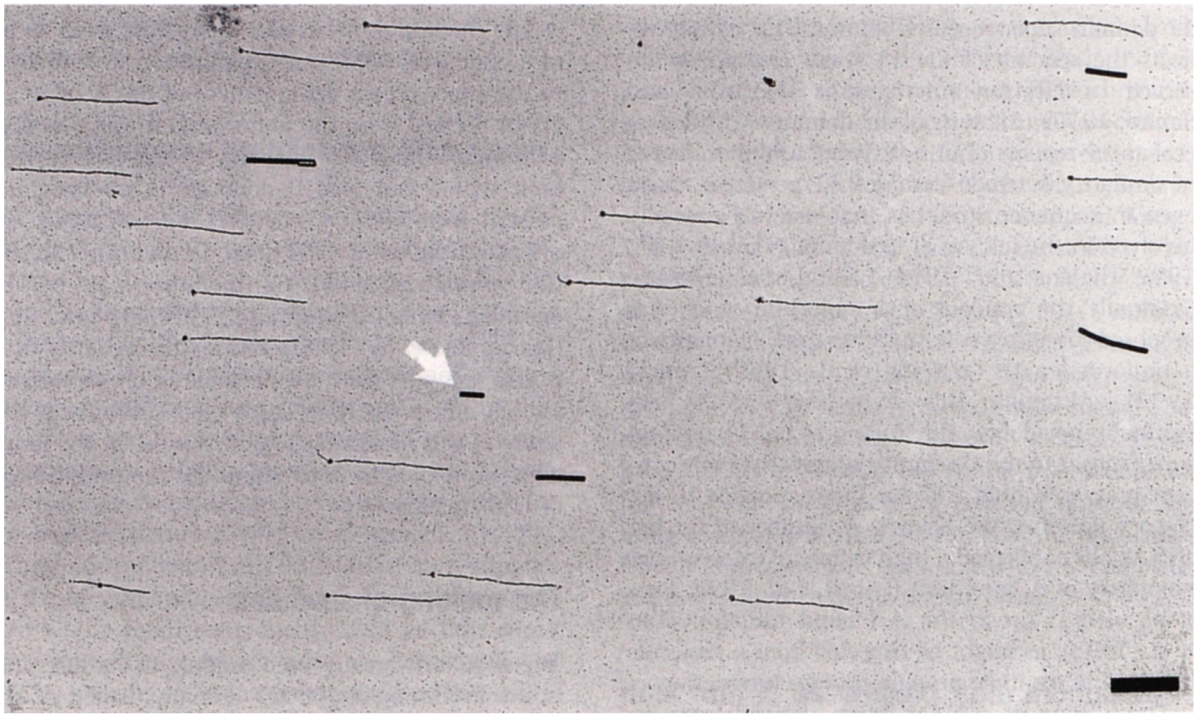

Figure 1 Morphology of titin molecules as revealed by low angle rotary shadowing with tantalum/tungsten. Note the uniform length of the molecules and the globular head-piece at the end that is located in the M band of the sarcomere. Tobacco mosaic virus was added for a comparison of sizes (arrow). Bar: 500 nm. (reproduced from Nave *et al.*, 1989).

integrity, then it has to be able to bind to a number of sarcomeric proteins at distinct parts of the myofibril. Titin must, for instance, be firmly attached to the thick filament. *In vitro* binding assays indeed indicated such a direct interaction (Maruyama *et al.*, 1985; Labeit *et al.*, 1992). A group of monoclonal titin antibodies exhibiting multiple epitopes in the A band also hinted at a more complex interaction of titin with the thick filament via C-protein and H-protein, both previously described as myosin-binding proteins (Fürst *et al.*, 1989b; Vaughan *et al.*, 1993 and references herein). This interaction has, at least for C-protein, been confirmed by various *in vitro* assays (Fürst *et al.*, 1992; Labeit *et al.*, 1992; Koretz *et al.*, 1993; Soteriou *et al.*, 1993b). The latter two papers also reported binding of titin to AMP-deaminase, an enzyme which is located in the I band of the sarcomere (Cooper and Trinick, 1984). It is interesting to imagine titin—beyond its role in anchoring the contractile apparatus—as a scaffold to immobilize certain enzymes or even multi-enzyme complexes (Cooper and Trinick, 1984).

Probably the strongest interactions of A band titin occur at the M disk: two major M band proteins, namely M-protein and myomesin, were shown to copurify consistently with titin under native conditions. The interaction could only be completely abolished by strong denaturants or by proteolysis (Nave *et al.*, 1989). An interesting aspect that is revealed by these observed bindings is the following: the results summarized above now put C-protein, H-protein, M-protein and myomesin into the context of a cytoskeletal framework. The recent availability of cDNA sequences for titin (see above), C-protein (Einheber and Fischman, 1990; Fürst *et al.*, 1992; Weber *et al.*, 1993), H-protein (Vaughan *et al.*, 1993), M-protein (Noguchi *et al.*, 1992; Vinkemeier *et al.*, 1993) and myomesin (Vinkemeier *et al.*, 1993) rather enforces this view. All these proteins, like titin, belong to the group of the intracellular members of the immunoglobulin superfamily (Fig. 2). Each "family member" is characterized by a particular pattern of immunoglobulin CII and fibronectin type III domains. M-protein and myomesin exhibit an overall sequence similarity of 50% and the same domain organization; thus they can be viewed as a subfamily (Vinkemeier *et al.*, 1993). The C-protein isoforms together with the H-protein seem to define another subfamily, since the domain sequence of the latter is identical to the C-terminal portion of C-proteins (Vaughan *et al.*, 1993). The distinct domain patterns may explain the differential localization within the A band of the sarcomere.

These findings now lead to a view of the cytoskeleton of the myofibril as a highly modular structure in which these two groups of domains build a structural framework for multiple protein-protein

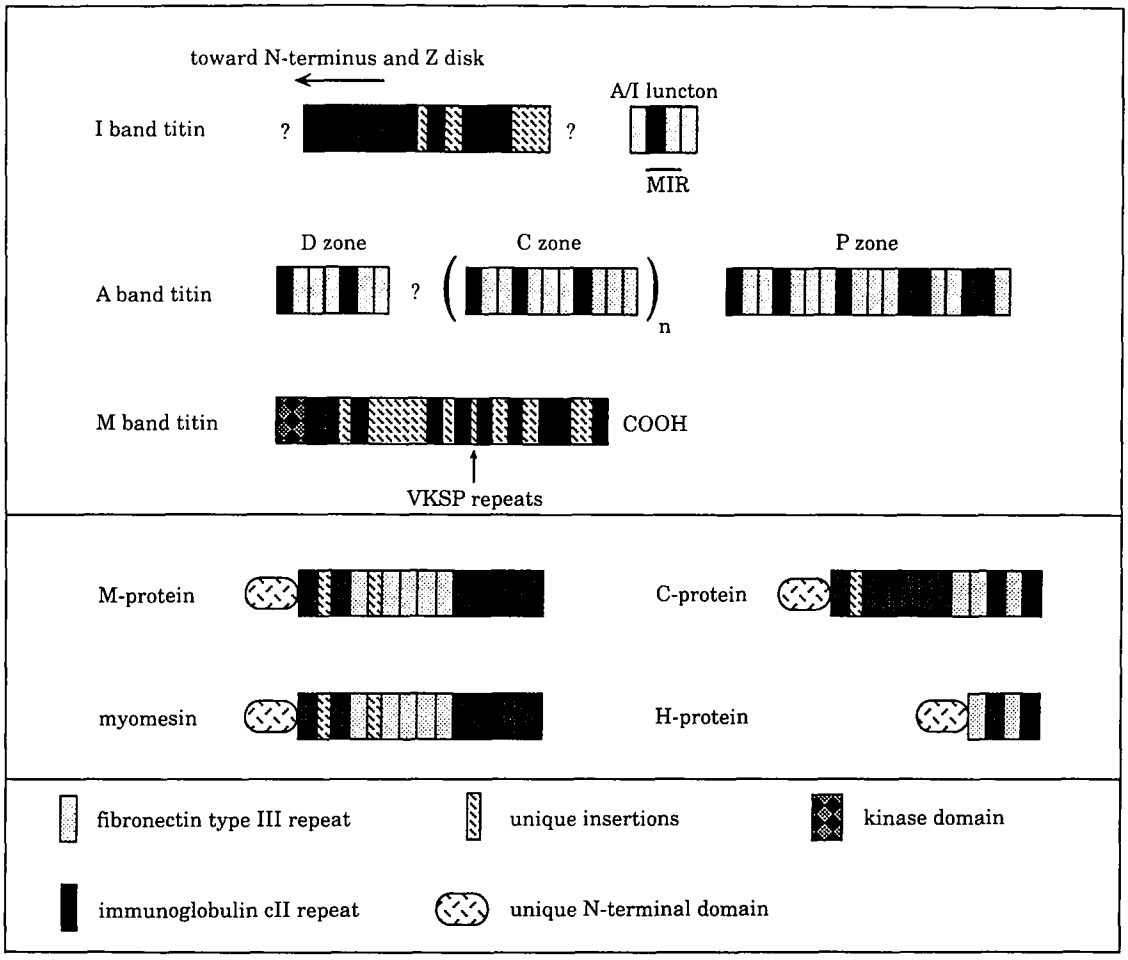

Figure 2 Domain organization of proteins comprising the cytoskeleton of the vertebrate striated myofibril. The schematic illustration of the repeat patterns of the cytoskeletal proteins identified so far reveals that they comprise to a common group, the intracellular members of the immunoglobulin superfamily (compiled from references cited in the text).

interactions. Homophilic, as well as heterophilic bindings of individual domains could occur in a similar fashion as in the extracellular proteins of the immunoglobulin superfamily. Individual domains could provide "a stable platform upon which a diversity of sequences are exposed on the external surfaces of the beta sheets or on the loops connecting the beta strands" (Williams *et al.*, 1989). The problem of structure–function correlation will certainly be tackled in the near future by assaying the interactions of a panel of bacterially expressed domains in combination with the ultrastructural mapping of functional domains by immunoelectron microscopy. This makes both a detailed structural and functional understanding of the sarcomeric cytoskeleton feasible.

The role of titin in muscular development

Morphogenesis of the striated myofibril involves a complex sequence of events which in the vertebrate embryo extends over a period of several days. The detailed analysis of the processes involved is complicated by the fact that heart muscle and the various skeletal muscles (trunk, head and limb muscles) develop on different time scales. The availability of muscle cell culture systems derived from embryonic tissue has considerably facilitated experimental access to these questions. The fundamental differences in the experimental systems used, however, has to be kept in mind when results are compared and/or extrapolated.

Several studies have investigated the expression and assembly of titin relative to other myofibrillar proteins (see also the review by Fulton and Isaacs, 1991). A first key question to be asked is, when does

the expression of the various sarcomere-specific proteins become evident? A series of studies using different combinations of antibodies, mainly on cultured cardiac myocytes, culminated in the assumption that the expression of desmin (the muscle-specific intermediate filament protein) in early post-mitotic myoblasts precedes the expression of a cohort of other myofibrillar proteins (see Schultheiss *et al.*, 1990 and references herein). The early appearance of desmin holds true also in several other studies on rat and mouse skeletal muscle (Bignami and Dahl, 1984; Fürst *et al.*, 1989a; Schaart *et al.*, 1989). A comparative analysis of the expression of sarcomeric proteins in the myogenesis of the mouse embryo, however, revealed a specific order of appearance: desmin→titin→α-actinin→muscle myosin, actin and myomesin→nebulin in myoblasts. M-protein and C-protein were only revealed much later when myotubes began to form (Fürst *et al.*, 1989a; Fürst and Weber, in preparation).

The next major event is the successive integration of these proteins into myofibrils, presumably on stress-fibre-like structures beneath the sarcolemma (Dlugosz *et al.*, 1984). In the early development of the chicken embryo heart, titin was found in periodically aligned spots together with α-actinin, at a time when sarcomeric actin and myosin were still diffusely organized (Tokuyasu and Maher, 1987). This could reflect the initiation of sarcomere formation at the Z line found earlier by electron microscopy (Markwald, 1973). Essentially the same conclusions were reached in studies of cultured chicken cardiomyocytes comparing the patterns of titin, actin and tropomyosin (Handel *et al.*, 1991) or those of titin, myosin, α-actinin and C-protein (Rhee *et al.*, 1994). On the other hand some reports concluded a close association of titin with myosin in developing muscle cells (see also review by Fulton and Isaacs, 1991). This discrepancy may be explained by the fact that the images provided by different titin antibodies depend on the position of the corresponding epitope along the titin polypeptide. Thus it seems that the Z line end is already anchored at an early stage (resulting in periodic labeling) whereas the interaction with the A band occurs at a later time point (Fürst *et al.*, 1989a). This view is strikingly supported by the recent characterization of the C-terminal portion of titin (see also above). This part of the molecule (situated in the M band of the sarcomere) is phosphorylated at early developmental stages by a kinase structurally related to cdc2 kinase. Upon differentiation these sites are likely to become dephosphorylated (Gautel *et al.*, 1993b). This would provide an elegant possibility to control the assembly of titin in the differentiating myofibril.

Titin and muscular disease

Although the large size of the titin gene and its repetitive structure seem to make it a likely target for genetic diseases, little is known so far about the involvement of the titin gene or protein in human disease. The invertebrate giant muscle proteins twitchin and projectin show several mutants with altered function. One mutant, for instance, results in anomalous movement in *C. elegans* (Benian *et al.*, 1989; Moerman *et al.*, 1988), while another one even leads to lethal phenotypes in homozygous *Drosophila* due to impaired hatching (Fyrberg *et al.*, 1992). No comparable genetic defects have however, been identified in human titin so far. One reason for this apparent scarcity of large genetic defects in the titin gene on human chromosome 2q (Labeit *et al.*, 1990) may be the crucial involvement of titin in muscle development, leading to early abortion due to a non-functional heart-anlage. On the other hand, small deletions or point-mutations may be of little effect due to the large size and redundancy of the modular arrays with multiple potentials for compensation, as long as continuity from the Z line to the M band is assured.

The known examples of titin in muscular disease seem to reflect mostly a secondary involvement, like an increased degradation of titin by calpain due to elevated calcium levels in Duchenne's dystrophy (Fürst *et al.*, 1987; Matsumura *et al.*, 1989). Surprisingly, titin was found to be specifically recognized as an autoantigen in a subset of Myasthenia gravis, where patients with myasthenia gravis and thymoma (MGT) form IgG autoantibodies against titin (Aarli *et al.*, 1987; Aarli *et al.*, 1990; Williams *et al.*, 1992). Molecular cloning using sera with high anti-titin titres demonstrated that these titin-autoantibodies are directed against a very short sequence stretch in the A/I junction, confined to a protein fragment of only about 30 kDa. (Gautel *et al.*, 1993a) which represents a main immunogenic region of titin. Assays using this recombinant titin fragment detect titin autoantibodies in 97% of patients with MGT. Clinically, the recognition of MGT is important since removal of a thymoma at an early stage may determine patient survival (LeGolvan and Bell, 1977). Hence, the availability of a recombinant antigen may prove a useful clinical marker for this purpose. Titin has also been described as a histological marker for the differential diagnosis of rhabdomyosarcoma *v* leiomyosarcoma and leiomyoma (Osborn *et al.*, 1986). In mice, the titin gene maps on proximal chromosome 2 in proximity to the gene of nebulin and the "muscular dystrophy

with myositis" (mdm) gene (Müller-Seitz *et al.*, 1993), but it is as yet unclear if this genetic disease is caused by mutations of either nebulin or titin or other genes. The complete cDNA sequence of titin will provide a basis to screen potential candidates at high resolution for mutations in the titin gene.

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