CELL STRUCTURE AND FUNCTION 22: 219–223 (1997)

Structural Interactions Responsible for the Assembly of the Troponin Complex on the Muscle Thin Filament

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ABSTRACT. **Skeletal muscle contraction is regulated by a complex of five polypeptides which are stably associated with the actin filament. This complex consists of two proteins: troponin with three subunits (TnC; TnI and TnT) and tropomyosin (a dimer of two chains). Using deletion mutants of TnC, TnI and TnT we determined that each of these polypeptides can be divided into at least two domains. One domain is responsible for the regulatory properties of the protein. Its interaction with the other components of the system change upon calcium binding to TnC. A second domain present in each of these proteins is responsible for the stable association of the complex to the actin filament. The interactions among this second set of domains is not influenced by calcium binding to TnC. The structural interactions are: 1) interactions between the C-domain of TnC with the N-domain of TnI; 2) interactions of the N-domain of TnI with the C-terminal domain of TnT and 3) interactions between the N-domain of TnT (T1) and actin/tropomyosin.**

Introduction

The molecular mechanisms involved in signal transduction pathways often involve one or more diffusable molecules which interact with their respective acceptor macromolecule. This interaction generally leads to a conformational change that regulates an enzyme activity. In most systems be binding of the diffusable molecule induces the conformational change. Examples include the assembly of transcription initiation complexes and the binding of calcium to calmodulin and the subsequent binding of calmodulin to different enzymes. Systems that rely on multiple diffusible macromolecules tend to present slow response times which are limited by diffusion. In skeletal muscle the result of evolutive pressure towards fast response times can be clearly observed. Diffusion is limited only to the calcium ions that leave the sarcoplasmic reticulum and bind TnC (18, 19, 20). In very fast muscles, the sarcoplasmic reticulum is positioned to reduce to a minimum the distances that the calcium ion has to diffuse before it reaches the thin filament. Calcium binding to TnC is the only diffusion limited step in the process. All the subsequent steps occur in the surface of the thin filament: a calcium induced conformational change on TnC which is transmitted to TnI, TnT and tropomyosin and finally activates seven actins along the filament. Furthermore each seven-actin regulatory unit is linked to the next unit by the tropomyosin overlap allowing a larger degree of cooperativity along the filament (2).

The fact that the regulatory machinery remains assembled on the surface of the filament during the whole regulatory cycle decreases the response time of the system but requires special design features. In systems where each component diffuses and binds to the next component, the energy involved in the binding process is used to induce the conformational change that carries the signal. In the thin filament, where all components are assembled all the time, we can imagine the system as a very large protein undergoing a complex allosteric conformational change, where the initial binding of the effector changes the conformation of the whole protein. If this is the case, how did the "allosteric" thin filament evolve from the slower and diffusion mediated system present in non-muscle cells and in smooth muscle cells?

The results we describe below indicate that although the thin filament can be described as an allosteric protein (1, 4, 7, 12), each of its components evolved to have structural elements which are involved in holding the filament assembled, while other elements change conformation and diffuse locally from one subunit to the next, carrying the information. Our results suggests that although the thin filament is a stable structure, it still retains some of the properties of the diffusion mediated systems (incomplete coupling for example) and some of the properties of an allosteric system (cooperativity for example).

Structural interactions between TnC and TnI

Since the original work of Potter and Gergely (25), it is known that only the two N-terminal metal binding sites of TnC are involved in the regulation of muscle contraction. The two sites present in the C-terminal domain bind both calcium and magnesium and are occupied by magnesium both in relaxed and confrecting muscle. It still remains to be determined if calcium does replace magnesium during tetanic contractions (19). It

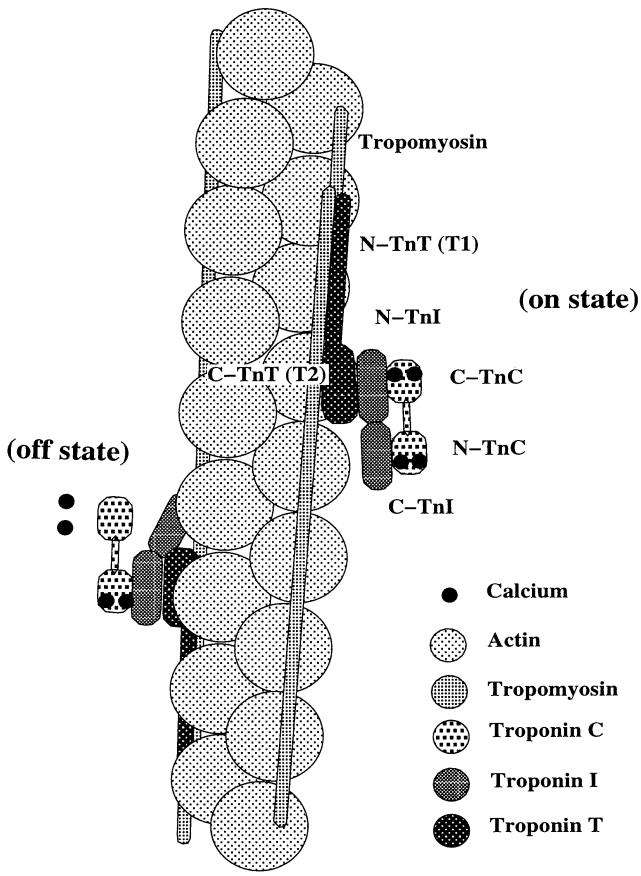

Fig. 1. The figure shows a diagram of the components of the thin filament and how their respective domains interact. The structural interactions described in the text are indicated. The C-domain of TnC (C-TnC) interacts with the N-domain of TnI (N-TnI). The N-domain of TnI interacts with the C-terminal domain of TnT (C-TnT) and the N-domain of TnT (N-TnT) interacts with tropomyosin. The left side of the figure shows the filament in the "off" state while the right side of the figure shows the filament in the "on" state.

has also been observed for a long time that the occupancy of the two C-terminal sites with metal ions are necessary for the maintenance of TnC in skinned muscle fibers (28). Recently it was demonstrated that mutations that destroy the C-terminal sites of TnC result in a TnC molecule that is still capable of regulating contraction but cannot be stably associated with the thin filament while mutations that destroy calcium binding to the amino-terminal sites abolishes regulation (26, 28). These results when analysed in the context of the crystal structure of TnC (16), which clearly indicates that each domain of TnC is structuraly independent suggests that the N-domain of TnC represents a regulatory domain while the C-domain is mainly a structural domain involved is maintaining TnC in the filament. These observations suggest that there must be a domain in the thin filament that anchors TnC.

Antiparallel organization of the TnC/TnI dimer. Troponin I, the inhibitory subunit of troponin has a central region responsible in large part for its inhibitory action (27, 29). Using a series of recombinant fragments of TnI, containing either the N-domain of TnI, the N-domain of TnI with the inhibitory region, the C-domain of TnI and the inhibitory region linked to the C-domain of TnI it was possible to map the interactions between TnC and TnI (8). It was demonstrated, using binding studies, that the N-domain of TnI interacts with the C-domain of TnC, while the C-domain of TnI interacts with the N-domain of TnC. As expected, these interactions could be significantly reduced if mutant TnCs containing non-functional metal binding sites were used in the binding assays (8). These results were supported by studies using deletion mutants of TnC where only the N-domain or only the C-domain were expressed. It was demonstrated that the N-domain of TnI interacts strongly with the C-domain of TnC, irrespective of the calcium concentration (in the presence of magnesium) while the N-terminal domain of TnC and the C-terminal plus inhibitory domain of TnI interacted in a calcium dependent fashion. When the regulatory effects of these combinations of fragments were studied it was demonstrated that the C-domain of TnI was necessary for regulation of the ATPase while the N-domain of TnI played a structural role (8).

These results led to a model for the TnC/TnI dimer were the two proteins are organized in an antiparallel fashion (8, 9). A natural consequence of this model is that the TnC/TnI dimer is also organized in a structural domain (N-terminal domain of TnI and the C-terminal domain of TnC) and a regulatory domain (inhibitory and C-terminal domain of TnI and the N-terminal domain of TnC). These results show that for the TnC/TnI dimer, the rule that the proteins are organized in separate structural and regulatory domains holds true (8).

It is known that in the absence of TnT, the TnI/TnC dimer can partially regulate the acto-myosin ATPase (3, 8). In the absence of calcium the dimer binds to the actin/tropomyosin filament (24) (by-way of the C-domain and the inhibitory region of TnI) but addition of calcium induces a conformational change in TnC which increases its affinity for the C-terminal region of TnI thereby sequestering this region of the molecule (9). This abolishes the interaction of TnI with actin/tropomyosin, releasing the TnC/TnI dimer from the filament and relieving the inhibition of the ATPase. The question therefore is how the TnC/TnI dimer is anchored to the actin/tropomyosin filament in the presence of calcium. Since this requires TnT, this property must reside in a fragment of TnT and in its counterpart in the TnC/TnI dimer.

Structural interactions between TnI and TnI

Deletion of the C-terminal region of TnT blocks the binding of TnC/TnI complex to TnT. Troponin T is an elongated molecule composed of approximately 260 residues. Its N-terminal domain extends along the C-terminal region of tropomyosin and is arranged in an antiparallel fashion in regard to the orientation of the tropomyosin molecule. A few but undetermined number of residues of TnT extends over the overlap region of tropomyosin (11). This elongated region of TnT corresponds approximately to the tryptic fragment T1. The C-terminal half of the TnT molecule is in contact with the TnC/TnI dimer and forms a globular structure that also binds near residue 190 of tropomyosin (11).

We have produced and characterized a series of deletion mutants of TnT (B. Malnic, Ph.D Thesis, 1995, University of São Paulo). A deletion of 50 residues in the C-terminal domain of TnT (residues 1–216) was not capable of retaining the TnC/TnI dimer bound to the actin/tropomyosin filament in the presence of calcium, although it was capable of binding strongly to the filament byway of its N-terminal domain. Conversely, a fragment of TnT containing only the last 60 residues of TnT (residues 194–263) was capable of binding strongly to the TnC/TnI dimer. This trimeric complex, containing TnC, TnI and this C-terminal fragment of TnT was not able to bind to actin/tropomyosin in the presence of calcium. These observations establish that the binding site of the TnC/TnI dimer to TnC resides in the last 50 residues of TnT. Furthermore we can conclude that this globular region containing TnC/TnI and the C-terminal part of TnT is anchored to the thin filament by way of the T1 region of TnT. We next investigated the region of the TnC/TnI dimer that interacts with the last 50 residues of TnT.

Deletions of the N-terminal of TnI blocks the binding of TnC/TnI complex to TnT. In order to map the domain of the TnC/TnI complex that interacts with the last 50 residues of TnT, we constructed dimers containing different deletion mutants of TnI (B. Malnic, Ph.D Thesis, 1995, University of São Paulo). These complexes were then analyzed for their binding to TnT. These experiments showed that only complexes where the N-terminal domain of TnI was absent lost their ability to bind to TnT and co-sediment with actin/tropomyosin (9). Reduced TnT incorporation into reconstitutes troponin lacking the amino terminal half of TnI has also been observed (8). TnI/TnC complexes containing deletions of the C-terminal half of TnI with or without the inhibitory region were still capable to bind TnT and sediment with the filaments. In a separate set of experiments we have also demonstrated that the reported observation that TnC interacts with TnT can be reproduced, but that the affinity between these two proteins is very low, being increased several fold by the addition of TnI. In conclusion these experiments indicate that the main interaction that maintains the TnC/TnI dimer bound to TnT occurs between the last fifty residues of TnT and the first 98 residues of TnI.

The structural core of the globular region of the troponin molecule

The binding experiments involving deletion mutants of TnC, TnI and TnT indicate that there are two major interactions that are responsible for holding together the three subunits. They are the interaction of the C-terminal half of TnC with the N-terminal half of TnI and the interaction of the last 50 residues of TnT with the N-terminal half of TnI. It is therefore clear that the N-terminal half of TnI plays a central role in maintaining the integrity of the globular domain of the troponin complex. The next question is how this globular domain is anchored to the thin filament.

The interaction of TnT/T1 with actin/tropomyosin

Experiments performed more than a decade ago with the triptic fragments of TnT (T1 and T2) have demonstrated that the region present in T1 interacts strongly with actin/tropomyosin (23, 30). This observation was confirmed using recombinant T1 in our laboratory. We have also demonstrated that T1, when bound to the thin filament, is not capable of holding either the TnC /TnI complex to the thin filament or the complex containing TnC, TnI and the C-terminal domain of TnT. These observations confirm the model proposed by Smillie and collaborators (15) in which the globular domain of the troponin complex interacts with the thin filament in the absence of calcium, but not in the presence of calcium. In the presence of calcium the globular domain remains associated with the filament only because it is linked to the long tail of T1 which is in turn bound to the filament. Experiments are in progress to further map te precise site of interactions between T1 and actin /tropomyosin. A question related to this specific interaction that is still not clarified is how the elongated T1 interacts with the filament. Does it interacts mainly with tropomyosin, or does it also interact with actin?

The role of the head-to-tail overlap of tropomyosin in the assembly of the tropomyosin/troponin complex onto actin filaments

The skeletal muscle tropomyosin is acetylated at its N-terminal residue. When this protein was first expressed in E. coli, it was observed that the recombinant form was not acetylated and that it also lacked some of the properties of the muscle protein (5, 14). The non acetylated tropomyosin lost its ability to polymerize in a head-to-tail fashion and showed a much reduced affinity for the actin filament. This lower affinity was not observed in the presence of whole troponin. These experi-

ments clearly demonstrated that the structure of the overlap region of tropomyosin is important for its assembly into the filament. It is important to note that although the head-to-tail overlap is the region spanned by the N-terminal region of TnT, it remains to be determined if this increase in affinity observed with troponin can be obtained with the T1 fragment of TnT. Using deletion mutants of TnT it has been demonstrated that TnT does influence the assembly of tropomyosin onto the actin filament, but not its calcium dependency (6, 10, 17). Two other lines of evidence confirmed the role of the head-to-tail overlap. First, in our laboratory we were able to construct tropomyosin molecules that were fully functional by including a two or three amino-acid fusions at the N-terminus (22). Second, studies using recombinant tropomyosins where the C-terminal exons were exchanged, demonstrated that some of these same properties were determined by their structure (13). All these results clearly indicate that there are interactions in the region of the head-to-tail overlap of two sequential tropomyosins which involve troponin T and which are also responsible for maintaining the structure of the thin filament.

Conclusion

These results demonstrate that there are clearly a set of interactions within the thin filament which are responsible for maintaining the structure of the filament. These interactions are not altered by calcium binding and are not directly involved in the process of regulation. A second set of interactions, not discussed here, are calcium dependent and involved in the regulatory process. Systems where the regulatory elements are not permanently assembled into a stable structure usually do not show a strong coupling between the different components of the system in a short time frame since the diffusion steps present during the transmission of the signal can delay the response. In contrast allosteric enzymes and complex enzyme systems usually show a highly coupled conformational change upon ligand binding. This is explained by the fact that the whole structure undergoes a concerted shift in conformation, and the interactions that hold the system in place are the same interactions that shift in response to ligand binding. In the case of the thin filament, a intermediate model may be in operation. Since there are separate sets of interactions to maintain the structure and to perform the regulation, this opens the possibility that a looser coupling might be observed in this structure. This would be observed as a longer delay in the spread of the binding signal along the filament when compared with the speeds observed in allosteric enzymes. This could be explained by the participation of a number of small range diffusion processes involving domains of the proteins within the complex. An example of such a process would be the movement of the inhibitory region of troponin I from troponin C towards the actin filament during inhibition. Further characterization of these processes in this system must await the development of conformation sensitive probes capable of detecting the spread of the signal from TnC to the other components of the system.

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