THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1989 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 264, No. 27, Issue of September 25, pp. 16299-16302, 1989
Printed in U.S.A.

Troponin I Switching in the Developing Heart*

(Received for publication, April 10, 1989)

Leopoldo Saggin, Luisa Gorza, Simonetta Ausoni, and Stefano Schiaffino

From the Institute of General Pathology and the Consiglio Nazionale della Ricerche Unit for Muscle Biology and Physiopathology, University of Padova, 35100 Padova, Italy

Monoclonal antibodies identify two distinct isoforms of troponin I in rat cardiac muscle, one predominant in the embryonic and fetal heart and one predominant in the adult heart. The two isoforms can be resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, with apparent molecular weights of 27,000 and 31,500, respectively. The adult isoform is specifically recognized by a monoclonal antibody that is unreactive with the embryonic variant, while two other monoclonal antibodies recognize both isoforms. A monoclonal antibody to cardiac troponin T was used to isolate by affinity chromatography the troponin complex from adult and neonatal rat heart. Affinity purified troponin from neonatal heart was found to contain both the embryonic and adult isoforms of troponin I. Comparative immunoblotting analysis with different muscle tissues shows that embryonic troponin I is identical with respect to electrophoretic mobility and pattern of immunoreactivity to the major troponin I isoform found in adult slow skeletal muscle. Troponin I switching may be implicated in developmental changes involving Ca^{2+} and pH sensitivity of the contractile system and response to β-adrenergic stimulation.

The troponin-tropomyosin complex is a Ca^{2+}-sensitive molecular switch that regulates actin-myosin interaction in striated muscle. Troponin consists of three subunits, troponin C (TnC),[1] troponin I (TnI), and troponin T (TnT). Binding of Ca^{2+} to TnC is thought to activate muscle contraction by releasing the inhibitory action of TnI-TnT-tropomyosin on actin-myosin interaction (1). Multiple forms of troponin subunits have been identified in fast skeletal, slow skeletal and cardiac muscle (2), and it appears that specific isoforms may play an important role in determining the distinctive functional properties of the various muscle types. Selective extraction of endogenous TnC from skinned cardiac trabeculae and its replacement with exogenous fast skeletal TnC was found to markedly reduce the sensitivity of the muscle to length change, suggesting that TnC isoforms are responsible for the difference in the response to stretching of skeletal and cardiac muscles (3). The Ca^{2+} sensitivity of the contractile system is determined not only by the type of TnC present but also by the other troponin subunits. Tobacman and Lee (4) have recently reported a differential effect of two cardiac TnT isoforms on the Ca^{2+} sensitivity of a reconstituted system of contractile proteins.

Developmental changes in functional properties of cardiac muscle, in particular those involving Ca^{2+} and pH sensitivity of the contractile system and response to β-adrenergic stimulation (5-8), may be related to troponin isoform switching. An embryonic-to-adult TnT switching has been clearly demonstrated in the avian and mammalian heart (9-14). In contrast, there are conflicting results concerning TnI switching. Humphreys and Cummins (15) found no evidence for changes in TnI composition in the developing bovine and human heart. On the other hand, other studies point to differences in TnI composition between fetal and adult heart (7, 8, 16-18).

We have investigated TnI changes in the developing heart using a set of monoclonal antibodies (mAbs) specific for TnI, TnT, and TnC. By immunoblotting and affinity chromatography procedures we have been able to identify a distinct TnI isoform which is transiently expressed in fetal and neonatal myocardium, and which appears to be similar to a major TnI isoform expressed in mature slow skeletal muscle.

MATERIALS AND METHODS

Protein Purification—Troponin was isolated from bovine atria or ventricles as described previously (12). An affinity chromatography procedure was used for the purification of troponin from adult and neonatal rat cardiac muscle. Tissue samples were homogenized in a low ionic strength buffer (20 mM KCl, 1 mM K_2HPO_4, 1 mM EGTA, pH 6.8) in the presence of protease inhibitors (0.5 μg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 1 μg/ml pepstatin). After centrifugation at 10,000 × *g* the pellet was extracted for 15 min with a high ionic strength buffer (40 mM sodium pyrophosphate, 1 mM $MgCl_2$, 1 mM EGTA, pH 9.0) containing the same protease inhibitors. The pyrophosphate extract, which is highly enriched in myofibrillar proteins, was applied to columns of CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology Inc.), that was conjugated with an anti-TNT mAb (12) following the instructions of the manufacturer. The columns were extensively washed with 10 mM Tris-HCl, pH 7.6, and bound proteins were eluted first with 0.5 M $MgCl_2$ in Tris buffer and then with 4 M $MgCl_2$ in Tris buffer. Elution with 0.5 M $MgCl_2$ yielded almost pure tropomyosin, whereas elution with 4 M $MgCl_2$ yielded almost pure troponin. The eluates were dialyzed against Tris buffer and used for SDS-polyacrylamide gel electrophoresis and immunoblotting analysis.

Monoclonal Antibodies—For the first fusion, which yielded mAb TI-1, Balb/c mice were immunized with a myofibrillar preparation from hypothyroid rabbit ventricle (12). For the second fusion, that yielded mAbs TI-3 and TI-4, mice were immunized with purified troponin from bovine heart. Cell fusion, screening and cloning of hybridomas, and mAb purification from ascites fluid were performed as described previously (12). Using class- and subclass-specific antisera we determined that TI-1 is an IgG_{2b} whereas both TI-3 and TI-4 are IgG_1. Two other antibodies were used in this study, one specific for cardiac TnT (RV-C2) (12), another specific for TnC (TC-1), that will be described in detail elsewhere.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—Immunoblotting analysis was performed on purified troponin and on whole tissue extracts prepared by homogenizing samples of bovine and rat cardiac or skeletal muscle in Laemmli buffer (19). Proteins were separated in SDS-12.5% polyacrylamide gels (19) and stained

* This work was supported by grants from the Ministero della Pubblica Istruzione and Consiglio Nazionale delle Ricerche (Gruppo Cardiorespiratorio, Grant 86.00448.04). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: TnC, troponin C; mAb, monoclonal antibody; TnT, troponin T; TnI, troponin I; SDS, sodium dodecyl sulfate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

with Coomassie Blue. For estimation of molecular weights a low molecular mass protein standard kit (Bio-Rad) was used. Duplicate gels were electrophoretically transferred to nitrocellulose sheets under conditions (2 h at 400 mA) that were found to allow complete elution of purified bovine troponins without significant transfer to a second sheet of nitrocellulose. The transfer was monitored with Coomassie Blue staining of the gel and Ponceau Red staining of the nitrocellulose. The blots were first incubated with anti-troponin antibodies, and then with peroxidase-conjugated rabbit anti-mouse Ig antibody (Dakopatts), and developed with diaminobenzidine in the presence of imidazole (20). Some blots were treated with alkaline phosphatase (Boehringer Mannheim) prior to antibody reaction as described previously (12).

RESULTS

Monoclonal Antibodies Identify a Distinct TnI Isoform in the Fetal Heart—The specificity of mAbs was determined by immunoblotting analysis on purified cardiac troponin and total tissue extracts form bovine heart. As shown in Fig. 1, the selected mAbs are specific for TnI. TnI is the only reactive component in total extracts of adult bovine ventricle; on the other hand, an additional band of higher electrophoretic mobility is recognized by mAbs TI-3 and TI-4 in extracts of bovine fetal heart.

Immunoblotting analysis on cardiac tissue from fetal, neonatal, and adult Wistar rats is illustrated in Fig. 2. The high mobility component (apparent molecular weight ~27,000) is predominant in the 16-day embryo (not shown) and in the 18-day fetus while the low mobility component (apparent molecular weight ~31,500) is predominant in the adult. The two components coexist in roughly similar amounts in ventricles from 7-day newborn rat. On immunoblots only the low mobility component is recognized by TI-1, whereas TI-3 and TI-4 react with both components. The pattern of reaction of TI-1 was unchanged by alkaline phosphatase pretreatment (data not shown). The reactivity of mAbs was further investigated by immunoblotting analysis using a variety of rat skeletal and cardiac muscle tissues, including extracts of a fast skeletal muscle, the tibialis anterior, and a slow skeletal muscle, the soleus (Fig. 3). The tibialis anterior muscle contains three varieties of type 2 fibers, type 2A, 2B, and 2X fibers, whereas the soleus muscle contains predominantly type 1 fibers with a minor type 2A component (21). Three bands of distinct electrophoretic mobility are recognized by mAb TI-4; a high mobility band present in fast skeletal muscle, an intermediate band present in slow skeletal muscle and in fetal

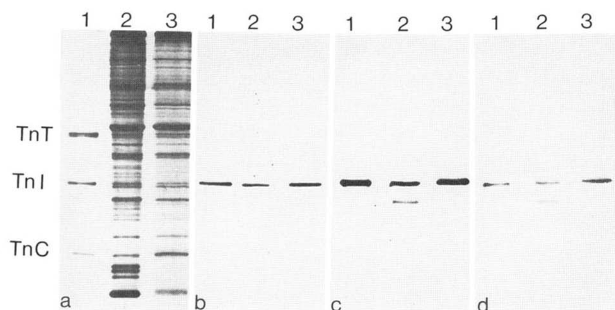

FIG. 1. **Immunoblot analysis of anti-TnI mAbs on purified troponin from adult bovine ventricles (*lane 1*) and total tissue extracts from 10-week fetal (*lane 2*) and adult (*lane 3*) bovine heart.** *a*, Coomassie Blue-stained gel. The positions of the TnT doublet, TnI and TnC are indicated at the *left*. *b–d*, corresponding immunoblots with mAbs TI-1 (*b*), TI-3 (*c*), and TI-4 (*d*).

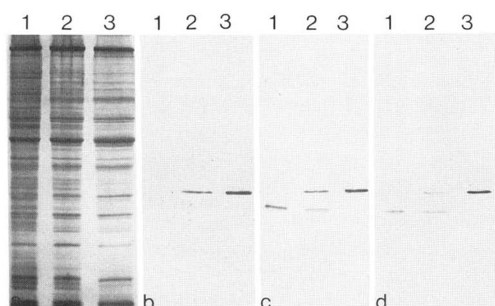

FIG. 2. **Immunoblots of total tissue extracts from 18-day fetal rat heart (*lane 1*), 7-day neonatal rat heart (*lane 2*), and adult rat heart (*lane 3*) with anti-TnI antibodies.** *a*, Coomassie Blue-stained gel. *b–d*, corresponding immunoblots with mAbs TI-1 (*b*), TI-3 (*c*), and TI-4 (*d*).

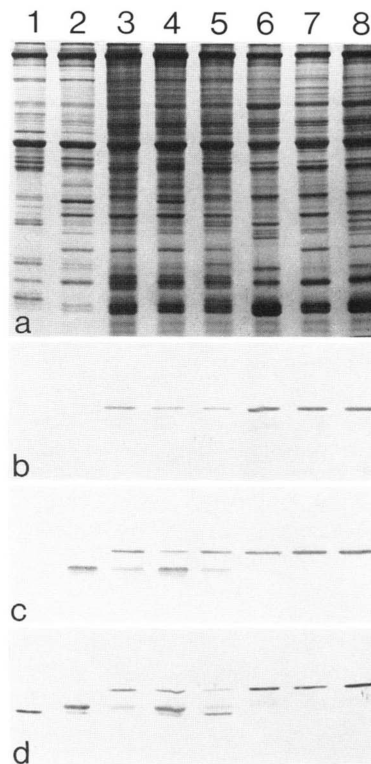

FIG. 3. **Immunoblots of total tissue extracts of different rat muscle tissues with anti-TnI antibodies.** *a*, Coomassie Blue-stained gel. *b–d*, corresponding immunoblots with mAbs TI-1 (*b*), TI-3 (*c*), and TI-4 (*d*). *Lane 1*, tibialis anterior muscle; *lane 2*, soleus muscle; *lane 3*, newborn (7-day-old) rat ventricle; *lane 4*, mixture of newborn ventricle with soleus; *lane 5*, mixture of newborn ventricle with tibialis; *lane 6*, adult atria; *lane 7*, adult ventricles; *lane 8*, mixture of atria and ventricles. Only the area of the blots corresponding to the position of TnI isoforms is shown. No other band outside this area was stained by the mAbs.

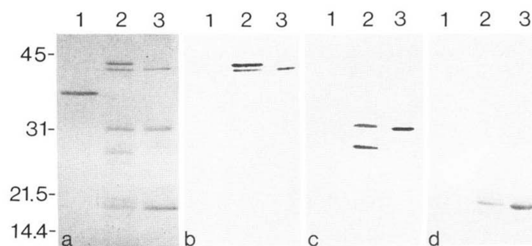

FIG. 4. **Isolation of tropomyosin from 30-day-old rat heart (*lane 1*) and troponin complex from 2-day-old (*lane 2*) and 30-day-old (*lane 3*) rat heart by affinity chromatography with insolubilized anti-TnT antibodies.** *a*, Coomassie Blue-stained gel of the proteins eluted with 0.5 M $MgCl_2$ (*lane 1*) and 4 M $MgCl_2$ (*lanes 2* and *3*) from anti-TnT-Sepharose. *b–d*, corresponding immunoblots with anti-TnT (*b*), anti-TnI (TI-4) (*c*), and anti-TnC (*d*). The position of molecular mass markers is indicated at the *left*.

cardiac muscle, and a low mobility band present in adult cardiac muscle. TI-3 shows a similar pattern of reactivity, except for lack of reactivity with fast skeletal TnI, whereas TI-1 reacts exclusively with adult cardiac TnI. A TnI band of identical electrophoretic mobility is identified by the mAbs in atrial and ventricular myocardium (Fig. 3, *lanes 6–8*). Fig. 3 also shows that soleus muscle extract contains an additional minor band that comigrates with the fast skeletal TnI of tibialis: this component may be due to the presence of type 2A fibers or might represent an additional slow skeletal isoform of TnI.

Purified Troponin from Newborn Rat Heart Contains the Fetal TnI Isoform—Results presented in the previous section suggest that embryonic and fetal heart contains a TnI isoform distinct from that present in the adult heart and similar to that present in slow skeletal muscle. However, the evidence is based only on immunologic reactivity and the possibility must be considered that the band recognized by our antibodies in the fetal heart is not TnI but an unrelated cross-reactive protein. For instance, it has recently been found that antibodies to TnT cross-react with glyceraldehyde-3-phosphate dehydrogenase (22). We first examined whether the binding of TI-3 and TI-4 to the embryonic component is weaker than to the adult TnI, as one might expect for a cross-reaction with an unrelated peptide (22). Immunoblotting experiments with neonatal rat heart using progressive dilutions of the antibodies showed no significant difference in the binding of the two antibodies to the embryonic and adult bands (not shown). To determine whether the postulated embryonic TnI is part of the troponin complex, we used an antibody specific for another troponin subunit, TnT, to isolate the troponin complex from neonatal heart. Crude myofibrillar extracts of adult or neonatal heart were applied to columns of CNBr-activated Sepharose conjugated with mAb RV-C2, which reacts with both TnT isoforms in bovine and rat heart (12). After washing with Tris-HCl 10 mM, tropomyosin was eluted with 0.5 M $MgCl_2$, then troponin was eluted with 4 M $MgCl_2$. The composition of cardiac troponin obtained by affinity chromatography is illustrated in Fig. 4. Whereas the adult preparation contains the typical three-subunit troponin complex, neonatal troponin consists of five major components: two TnTs, corresponding to the embryonic and adult isoforms (12), two TnIs corresponding to the adult and the postulated embryonic isoforms, and one TnC undistinguishable by electrophoretic mobility from adult TnC. The finding that the high mobility band recognized by TI-3 and TI-4 in neonatal heart is retained by immobilized anti-TnT together with the other subunits of the troponin complex, strongly supports the view that this band is in fact a troponin subunit and not a cross-reactive peptide. Identification of this band as a fetal TnI isoform is consistent with the finding that it is recognized by antibodies to TnI and not by antibodies to TnT and TnC (Fig. 4, *b–d*).

DISCUSSION

The results of the present study indicate that two antigenically and electrophoretically different isoforms of TnI are expressed during cardiac development. Embryonic TnI has a lower apparent molecular weight than adult TnI in both rat and bovine heart. This result is in agreement with a recent report on TnI changes in rat and chicken heart (18). The TnI isoform predominantly expressed in the fetal heart appears to be similar in electrophoretic mobility and antigenic properties to the major TnI isoform expressed in slow skeletal muscle. In this respect, pending definitive evidence for the identity of slow skeletal TnI with embryonic cardiac TnI, TnI switching is reminiscent of myosin heavy chain switching in the rat heart (23): in both cases there is transient expression in the developing heart of contractile protein isoforms (β-myosin heavy chain and slow TnI, respectively) which are expressed as definitive forms in slow skeletal muscle. We have previously reported that TnT also undergoes an embryonic-to-adult isoform transition in rat cardiac muscle (12). TnI and TnT switching occurs in the perinatal period and is essentially completed in the first month after birth.

The finding that fetal heart contains a TnI isoform different from that present in the adult heart and similar to that expressed in slow skeletal muscle, may have important implications for the contractility of cardiac muscle and in particular for the changes in contractility induced by inotropic agents. Cardiac TnI is larger than skeletal TnIs due to the presence of additional 27–33 residues at the amino-terminal end of the molecule (24, 25). A serine residue located in this portion of the molecule is specifically phosphorylated when hearts are perfused with β-adrenergic agonists (26, 27) or when troponin preparations are incubated *in vitro* with cyclic AMP-dependent protein kinase (28, 29). Phosphorylation of TnI at this site results in a decreased affinity of troponin C for Ca^{2+} and a rightward shift of the relationship between Ca^{2+} and myofibrillar force (30) and might contribute to the more rapid relaxation induced by β-adrenergic agonists (see Ref. 31 for a review).

Both fast skeletal and slow skeletal TnI lack the amino-terminal extension of cardiac TnI, therefore do not show the phosphorylation-mediated regulation of Ca^{2+} sensitivity by β-adrenergic agents. The results presented here suggest that the same may be true for the TnI isoform present in the fetal heart. Newborn rabbit and dog myocardium is less sensitive to isoproterenol than adult myocardium (6, 32). In particular, the relaxation time does not shorten with isoproterenol in the newborn like in the adult heart: this may be related not only to the poor development and reduced calcium-accumulating capacity of the sarcoplasmic reticulum (33, 34) but also to the different TnI composition. It will be of interest to reinvestigate the effect of inotropic agents in the developing heart in relation to the changing composition of the troponin complex.

TnI switching may also be implicated in the developmental changes in Ca^{2+} sensitivity of cardiac myofilaments and specifically in the differential effects of pH on Ca^{2+} activation of myofilaments from adult and neonatal hearts (5, 7, 8). Ca^{2+} activation of myofilaments is unaffected (7) or only slightly affected (8) by a drop in pH from 7.0 to 6.5 in the fetal and neonatal mammalian heart, whereas in the adult heart the same drop in pH causes a marked rightward shift in the relation between pC_a and myofibrillar ATPase. This effect appears to be due to a reduction in the Ca^{2+}-binding affinity of TnC and has been related to the possible presence of distinct TnI variants in the fetal heart (7, 8). A role of TnI in this respect would be consistent with the finding that the depression of Ca^{2+} binding to skeletal TnC induced by low pH is enhanced by TnI as a result of a decrease in the affinity of TnI for TnC at acidic pH (35).

REFERENCES

1. Ebashi, S., and Endo, M. (1972) *Prog. Biophys. Mol. Biol.* **64,** 465–477
2. Perry, S. V. (1986) in *Myology: Basic and Clinical* (Engel, E. G., and Banker, B. K., eds) pp. 613–641, McGraw-Hill, New York
3. Babu, A., Sonnenblick, E., and Gulati, J. (1988) *Science* **240,** 74–76
4. Tobacman, L. S., and Lee, R. (1987) *J. Biol. Chem.* **262,** 4059–4064
5. Fabiato, A. (1982) *Fed. Proc.* **41,** 2238–2244
6. Park, I. S., Michael, L. H., and Driscoll, D. J. (1982) *Am. J. Physiol.* **242,** H13–H18

7. Solaro, R. J., Kumar, P., Blanchard, E. M., and Martin, A. F. (1986) *Circ. Res.* **58,** 721–729
8. Solaro, R. J., Lee, J. A., Kentish, J. C., and Allen, D. G. (1988) *Circ. Res.* **63,** 779–787
9. Cooper, T. A., and Ordahl, C. P. (1985) *J. Biol. Chem.* **260,** 11140–11148
10. Ogasawara, Y., Komiya, T., and Obinata, T. (1987) *J. Biochem. (Tokyo)* **102,** 25–30
11. Jin, J.-P., and Lin, J. J.-C. (1988) *J. Biol. Chem.* **263,** 7309–7315
12. Saggin, L., Ausoni, S., Gorza, L., Sartore, S., and Schiaffino, S. (1988) *J. Biol. Chem.* **263,** 18488–18492
13. Anderson, P. A. W., Moore, G. E., and Nassar, R. N. (1988) *Circ. Res.* **63,** 742–747
14. Sabry, M. A., and Dhoot, G. K. (1989) *J. Mol. Cell. Cardiol.* **21,** 85–91
15. Humphreys, J. E., and Cummins, P. (1984) *J. Mol. Cell Cardiol.* **16,** 643–657
16. Toyota, N., and Shimada, Y. (1981) *J. Cell Biol.* **91,** 497–504
17. Nakanishi, T., Nagae, M., and Takao, A. (1986) *Circ. Res.* **58,** 890–895
18. Sabry, M. A., and Dhoot, G. K. (1989) *J. Muscle Res. Cell Motil.* **10,** 85–91
19. Laemmli, U. K. (1970) *Nature* **227,** 680–685
20. Trojanowski, J. Q., Obrocka, M. A., and Lee, V. M.-Y. (1983) *J. Histochem. Cytochem.* **31,** 1217–1223
21. Schiaffino, S., Gorza, L., Sartore, S., Saggin, L., Ausoni, S., Vianello, M., Gundersen, K., and Lømo, T. (1989) *J. Muscle Res. Cell Motil.* **10,** 197–205
22. Sanders, C., Stewart, D. I. H., and Smillie, L. B. (1987) *J. Muscle Res. Cell Motil.* **8,** 118–124
23. Hoh, J. F. Y., McGrath, P. A., and Hale, H. T. (1978) *J. Mol. Cell Cardiol.* **10,** 1053–1076
24. Wilkinson, J. M., and Grand, R. J. A. (1978) *Nature* **271,** 31–35
25. Leszyk, J., Dumaswala, R., Potter, J. D., and Collins, J. H. (1988) *Biochemistry* **27,** 2821–2827
26. England, P. J. (1976) *Biochem. J.* **160,** 295–304
27. Solaro, R. J., Moir, A. J. G., and Perry, S. V. (1976) *Nature* **262,** 615–617
28. Moir, A. J. G., Cole, H. A., and Perry, S. V. (1977) *Biochem. J.* **161,** 371–382
29. Stull, J. T., and Buss, J. E. (1977) *J. Biol. Chem.* **252,** 851–857
30. Mope, L., McClellan, G. B., and Winegrad, S. (1980) *J. Gen. Physiol.* **75,** 271–282
31. Winegrad, S. (1984) *Circ. Res.* **55,** 565–574
32. Park, M. K., Sheridan, P. H., Morgan, W. W., and Beck, N. (1980) *Dev. Pharmacol. Ther.* **1,** 70–82
33. Page, E., and Buecker, J. L. (1981) *Circ. Res.* **48,** 519–522
34. Nayler, W. G., and Fassold, E. (1977) *Cardiovasc. Res.* **11,** 231–237
35. El-Saleh, S. C., and Solaro, R. J. (1988) *J. Biol. Chem.* **263,** 3274–3278