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Isolation of human cardiac troponin T and localization of epitopes recognized by monoclonal antibodies to cardiac troponin T

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Human cardiac troponin T has been isolated and its properties compared with those of rabbit skeletal and bovine cardiac troponin T. Seven monoclonal antibodies to troponin T have been obtained. Two antibodies cross-reacted with both cardiac and skeletal troponin T and recognized epitopes located between residues 98–177 of bovine cardiac troponin T. Five other antibodies were specific for cardiac troponin T and recognized antigenic determinants located between residues 180–258 of bovine cardiac troponin T. Localization of antigenic determinants in the central part of troponin T seems to be due to the high hydrophilicity and flexibility of this part of the molecule. The monoclonal antibodies thus obtained may be used for diagnosing various types of human heart diseases.

Cardiac troponin T; Phosphorylation; Monoclonal antibody; Heart disease

1. INTRODUCTION

The troponin–tropomyosin complex is involved in the regulation of actin–myosin interactions in skeletal and cardiac muscles [1,2]. Troponin T, one of the three troponin components, is a structural protein interacting with actin and tropomyosin [1,2]. There are no less than three genes coding troponin T of fast and slow skeletal and heart muscle [3]. Troponin T mRNA undergoes alternative splicing which leads to the formation of a great number of troponin T isoforms [4,5]. Recently obtained antibodies specifically interacting with human cardiac troponin T were successfully used for the detection of heart damage [6,7]. The primary structure of human cardiac and skeletal troponin T is still unknown. This paper deals with the isolation of preparative quantities of human cardiac troponin T and preparation and characterization of monoclonal antibodies against this protein.

2. MATERIALS AND METHODS

Rabbit skeletal and bovine cardiac troponin T were isolated as described earlier [8,9]. The method of human cardiac troponin T purification consists of extraction, heat treatment, ammonium sulphate fractionation and ion-exchange chromatography. The details of purification will be published elsewhere. Isolated troponin T was phosphorylated by casein kinase II from rat liver [10] or by phosphorylase kinase from rabbit skeletal muscle [11] kindly provided by Dr. P.L. Vulfson (Department of Biochemistry, Moscow State University). The phosphorylation conditions were similar to those described earlier [8].

To localize antigenic determinants, troponin T phosphorylated by one of the protein kinases mentioned above was either cleaved by CNBr [12] or subjected to limited chymotrypsinolysis [13]. Peptides thus obtained were subjected to SDS-gel electrophoresis [14,15]. After staining with Coomassie R-250, the gels were dried and autoradiographed.

Monoclonal antibodies were obtained by the method of Kohler and Milshtein [16] using bovine cardiac and human cardiac troponin T as antigens. Antibody isotypes were determined using the Calbiochem kit. Screening of the primary clones and determination of the antibody specificity was performed by the solid-phase immunoenzyme method, with rabbit skeletal, bovine, and human cardiac troponin T as antigens. After electrophoresis troponin T and its peptides were transferred to nitrocellulose filters by semi-dry blotting [17]. The nitrocellulose filters were incubated with ascite fluids at a 1:1,000 dilution for 1 h and after washing treated with anti-mouse antibodies conjugated with peroxidase. The blots were stained in β-naphtol-containing medium.

Determination of sites with the highest hydrophilicity and highest flexibility within the structure of cardiac troponin T was performed by the PC Gene program (subprograms Flexpro and Antigen).

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3. RESULTS

3.1. *Isolation of human cardiac troponin T and investigation of its properties*

The method developed allows homogeneous troponin T to be obtained (see Fig. 1). We were unable to detect human cardiac troponin T isoforms by either ion-exchange chromatography or SDS-gel electrophoresis. These data agree well with the results of Anderson et al. [18] which indicated that normal human hearts predominantly contain one isoform of troponin T.

We compared the structures of troponin T isolated from bovine and human heart and rabbit skeletal mus-

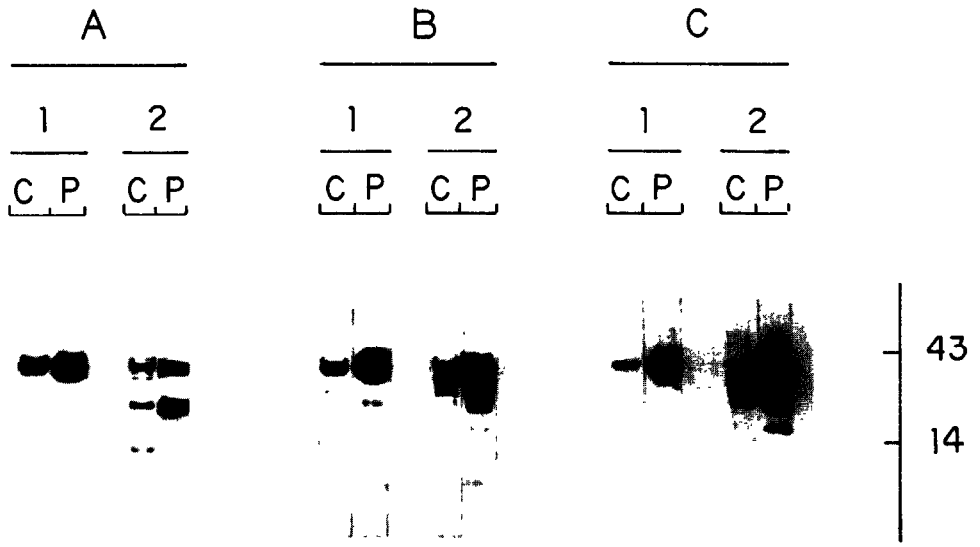

Fig. 1. Electrophoregrams (c) and autoradiograms (p) of native troponin T (1) and its chymotryptic peptides (2). A, B and C are rabbit skeletal, bovine and human cardiac troponin T, respectively. On the right is a scale of apparent molecular weights.

cle using one-dimensional peptide mapping. Chymotrypsinolysis of rabbit skeletal troponin T produced two fragments, i.e. TnT_1, containing residues 1–158 (M_r 23 kDa), and TnT_2, containing residues 159–259 (M_r 14 kDa) (Fig. 1). Casein kinase II phosphorylates only Ser-1 of both cardiac and skeletal troponin T [8], therefore only TnT_1 contains the radioactive phosphate (Figs. 1A, 2). One-dimensional chymotryptic peptide maps of human and bovine cardiac troponins T are similar (cf. Fig. 1B,C) but differ from those of rabbit skeletal troponin T. Chymotrypsinolysis of cardiac troponin T produces a number of N-terminal peptides with M_r in the range of 35–24 kDa, containing the site phosphorylated by casein kinase II (Fig. 1B,C), but no peptides with M_r 14 kDa, analogous to the C-terminal TnT_2 fragment of skeletal troponin T. The pattern of CNBr-peptides of bovine and human cardiac troponin T is also very similar. The only difference is that human cardiac troponin T contains a very short (M_r < 4 kDa) CNBr peptide which is absent in the case of bovine cardiac troponin T. This peptide contains sites phosphorylated by casein kinase II; therefore we presume that human cardiac troponin T contains an additional Met residue close to the N-terminus which is absent in the primary structure of bovine cardiac troponin T.

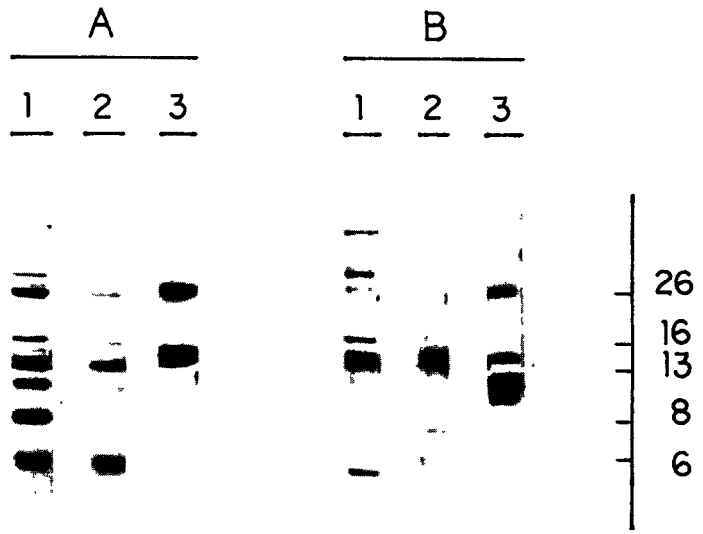

Fig. 2. Electrophoretic separation of CNBr-peptides of rabbit skeletal (A) and bovine cardiac troponins T (B). Electrophoresis was performed by the method of Schagger and von Jagow [16]. Lane I, gels stained with Coomassie R-250; lanes 2 and 3, autoradiograms after electrophoresis of CNBr-peptides of troponin T phosphorylated by phosphorylase kinase and casein kinase II, respectively. On the right is a scale of apparent molecular weights.

3.2. *Identification of troponin T peptides on SDS-polyacrylamide gels*

Since the structure of bovine cardiac troponin T is similar to that of human cardiac troponin T, the greater part of this investigation was performed on rabbit skeletal and bovine cardiac troponin T. Cleavage of rabbit skeletal troponin T with CNBr resulted in the formation of peptides of M_r 26, 24, 16, 13.5, 10.5, 8.0, and 5.9 kDa. The bands migrating with M_r 13.5, 8.0 and 5.9 are doublets. Two CNBr-peptides of troponin T, CB5 (residues 152–175) and CB6 (residues 239–259), migrate in the region of M_r 5.9 kDa. One of these peptides, CB5, contains sites (Ser-156/Ser-157) phosphorylated by phosphorylase kinase [12] (Fig. 2A2). The 8 kDa peptide does not contain any sites of phosphorylation and corresponds to the CB4 peptide (residues 176–230) of rabbit skeletal troponin T. The 10.5 kDa peptide contains sites phosphorylated by phosphorylase kinase and, according to its size, corresponds to the uncleaved fragment, including peptides CB5 and CB4 (residues 152–230). The lower band of M_r 13.5 kDa contains sites phosphorylated by phosphorylase kinase and corresponds to the CB2 peptide (residues 71–151). The peptide of a slightly higher M_r, badly stained with Coomassie R-250, contains sites phosphorylated by casein kinase (Fig. 2A3) and corresponds to peptide CB3 (residues 1–70) of rabbit skeletal troponin T. All other high molecular weight peptides are the products of incomplete digestion. The pattern of CNBr-peptides and their relative electrophoretic mobilities correlate well with the data of [19].

CNBr-peptide maps of bovine cardiac troponin T are far less informative. Usually we detected the bands of M_r 28, 16.5, 13.2–13.6, 11 and 5.2 kDa on the gels. The smallest peptide of M_r 5.2 kDa does not contain phosphorylation sites and is identical to CB2 peptide (residues 69–92) of bovine cardiac troponin T [20]. A badly stained peptide of M_r 11 kDa contains the sites phosphorylated by casein kinase II and seems to correspond to the CB1 peptide (residues 1–68). The peptides migrating in the region of 13.2–13.6 kDa contain sites phosphorylated by both kinases (Fig. 2B2,3). We presume that this region contains peptide CB3 (residues 93–178, including Ser-176 which is phosphorylated by phosphorylase kinase), a partly hydrolyzed fragment containing residues 1–92 (peptides CB1–CB2 with Ser-1 phosphorylated by casein kinase II), as well as the CB4 peptide (residues 180–284) which is not phosphorylated by either protein kinases. The other peptides of higher M_r are the products of incomplete digestion.

3.3. *Localization of epitopes recognized by monoclonal antibodies within the structure of troponin T*

After primary screening we selected seven clones producing antibodies effectively reacting with cardiac troponin T. Two clones, 11DE4 and 4D11, produced antibodies which recognize both skeletal and cardiac troponin T (Table I). These antibodies stained CNBr-peptides of troponin T with M_r > 13 kDa (Fig. 3A) and did not interact with either CB1 (M_r 11 kDa) or CB2 (M_r 5.2) peptides of cardiac troponin T. The data presented indicate that these antibodies interact with the epitope located between residues 71–151 of rabbit skeletal troponin T or in the homologous region (residues 98–178) of bovine cardiac troponin T.

Table I

Some properties and the specificity of monoclonal antibodies to troponin T

Clone	Ig type	Specificity for troponin T			Localization of epitope in bovine cardiac troponin T
		Bovine cardiac	Human cardiac	Rabbit skeletal	
4D11	IgG_{2b}	100	105	105	98–178
11DE4	IgG_{2b}	100	120	120	98–178
2EG11	IgG_{2a}	100	75	5	180–258
11GD5	IgG_{2a}	100	120	4	180–258
5C12	IgM	100	125	9	180-258
4C11	IgM	100	60	8	204–258
1F2	IgG_1	100	105	4	204–258

The specificity was determined by the ELISA method, the intensity of bovine cardiac troponin T staining was taken for 100%.

Antibodies 2EG11, 11GD5 and 5C12 specifically interact with cardiac troponin T (Table I). These antibodies weakly stained CNBr-peptides of skeletal troponin T with M_r 10.5 kDa (CB5–CB4, residues 152–230) and effectively interacted with the homologous region of cardiac troponin T (residues 180–258) included in the CB4 peptide with M_r 13 kDa (Fig. 3B).

Antibodies 1F2 and 4C11 are also specific to cardiac troponin T (Table I). These antibodies weakly stained TnT_2 and CNBr-peptides (M_r 10.5 and 8.0 kDa) of skeletal troponin T and interacted with a 13 kDa CNBr-peptide of cardiac troponin T (Fig. 3C). This means that antibodies 1F2 and 4C11 interact with the epitope located between residues 176–230 of skeletal troponin T and the homologous fragment of cardiac troponin T (residues 204–258).

4. DISCUSSION

There are only a few preliminary reports on the preparation of monoclonal antibodies against cardiac troponin T [6,7,21]. Katus and co-workers [6,7,22] developed a sensitive and reliable immunological method for determination of cardiac troponin T in the blood. However, there were no data on the localization of epitopes recognized by cardiac troponin T antibodies. Therefore we prepared a panel of monoclonal antibodies and determined the epitopes recognized by these antibodies in cardiac and skeletal troponin T. To our surprise all antibodies predominantly interact with the central part

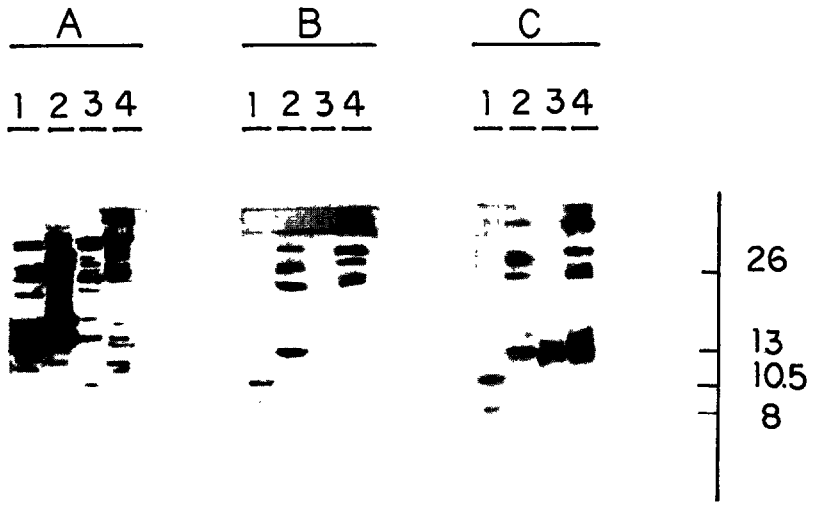

Fig. 3. Immunoblots after staining of CNBr (lanes 1,2) and chymotryptic (lanes 3,4) peptides of rabbit skeletal (lanes 1,3) and bovine cardiac (lanes 2,4) troponin T with monoclonal antibodies 11ED4 (A), 2EG11 (B) and 1F2 (C).

of the troponin T molecule (Table I). To explain the uneven distribution of antigenic determinants we analyzed the primary structure of bovine cardiac troponin T using the PC Gene program. The peptides containing residues 110–116, 140–145, 155–160, and 200–206, are the most hydrophilic and the regions restricted by residues 44–50, 109–115, 157–163, 192–198 and 200–206 are characterized by high flexibility. Hydrophilic and flexible regions located on the protein surface are, as a rule, highly antigenic [23], therefore it is no surprise that all our antibodies predominantly interact with the part of the cardiac troponin T molecule restricted by residues 98–258.

In future we are planning to use the antibodies obtained for determining cardiac troponin T in serum with its further application in the diagnosis of myocardial damage.

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