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TRANSGLUTAMINASE-CATALYZED POLYMERIZATION OF TROPONIN IN VITRO

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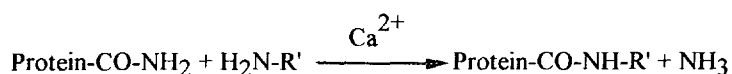
Summary In the presence of calcium ions, tissue transglutaminase catalyzes the polymerization of skeletal muscle troponin to high molecular weight insoluble aggregate. The specific action of transglutaminase is proved by the isolation of glutamyl-spermidine isopeptide derivatives. The process involves mainly the troponin T subunit (TnT), with formation of dimers and trimers of TnT, which were reactive with specific antibodies by immunoblotting. Furthermore when incubation is carried out in the presence of radioactive polyamines, the label is incorporated selectively into TnT subunits. © 1995 Academic Press, Inc.

Transglutaminases catalyze the posttranslational modification of proteins leading to crosslinkage or covalent incorporation of primary amines through isopeptide bonds at selected glutamine residues [1]. The reaction is strictly dependent on the presence of high concentration of calcium ions, so that the enzyme is latent under normal conditions.



$$\text{Protein-CO-NH}_2 + \text{H}_2\text{N-R}' \xrightarrow{Ca^{2+}} \text{Protein-CO-NH-R}' + \text{NH}_3$$

Proteins acting as substrates for type 2 transglutaminases in situ include (i) several cell surface and matrix associated proteins (fibrinogen, osteopontin [2], fibronectin and their receptors, nidogen and laminin, vitronectin and possibly type III collagen [reviewed in ref. 3]); (ii) cellular fibrillar proteins, as cytokeratins [4] and involucrin-like proteins [5], and (iii) the contractile proteins myosin and actin [6, 7] and other myofibrillar proteins anchored to the Z-line [8]. In vitro studies suggested that contractile and cytoskeletal proteins are preferred substrates of transglutaminases [6]. A major difficulty in identification of potential substrates is represented by the highly aggregated state of the products which are hardly resolved by SDS-PAGE [9]. Identification of proteins

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acted upon by transglutaminases is pivotal in the understanding of their involvement in either apoptosis or necrosis. In fact, it has been proposed that transglutaminases are involved in cell death programs, as terminal differentiation of keratinocytes and apoptosis [10,11] or in ischemic cell death [4]. Accordingly, rapid enzyme activation takes place following marked increase in cytosolic calcium ($[Ca^{2+}]_c$), leading to irreversible changes in cell structure, protein aggregation and production of apoptotic bodies, subsequently cleared by phagocytosis [12]. Alternatively, TGase activation in tissues, such as skeletal or cardiac muscle, which do not usually undergo apoptosis might participate in evolution towards cell necrosis. In this perspective, this report demonstrates that TnT subunit of skeletal muscle is a substrate for transglutaminase in vitro.

MATERIALS AND METHODS

Protein purification. Human erythrocyte trangslutaminase was purified by affinity chromatography on heparin-sepharose, with an additional ion-exchange chromatography step [13], to remove trace contaminants. Rabbit skeletal muscle troponin complex, a kind gift of prof. E. Grazi from our laboratory, was extracted from muscle ethanol-ethylether powder, and purified by isoelectric precipitation of tropomyosin, ammonium sulfate fractionation and chromatography on Blue-sepharose [14].

Modification of troponin by transglutaminase. Troponin, diluted to 1-1.5 mg/ml with 50 mM Tris buffer pH 7.8, was incubated with purified transglutaminase (20 μg/ml) in the presence of 5 mM Ca++ ions for increasing periods of time, before removing samples for SDS-PAGE. In some instances, we added also radioactive putrescine (0.6 mM, 4000 dpm/pmole) to estabilish an apparent stoichiometry of covalent binding. In this case, samples were removed, spotted onto squares of filter paper, extensively washed with cold TCA and counted for protein-bound radioactivity, exactly as in the usual assay of transglutaminase activity [15].

Identification of the products of transglutaminase catalized reaction between troponin and [^{3}H] spermidine. Purified troponin (3.7 mg/ml) was incubated with 400 µM [^{3}H] spermidine in the presence of erythrocyte transglutaminase for two hours at room temperature, followed by lyophilization [2]. Aliquots of the lyophilized samples were cleared of free [^{3}H] spermidine [16] and solubilized in 0.2 M N-ethyl morpholine acetate buffer pH 8.1. Proteins were extensively digested with proteinases and analyzed for γ-glutamyl [^{3}H] spermidine by ion- exchange chromatography as described [17]

Electrophoretic procedures. At the required time of incubation samples were removed, supplemented with an equal volume of 125 mM Tris.HCl, 0.2% SDS and 20 mM mercaptoethanol, pH 6.8, boiled and electrophoresed on 12% or on 6-16% linear gradient acrylamide slab-gels, according to the procedure of Laemmli [18]. The gels were either stained with Coomassie Brilliant Blue R-250 or transferred to nitrocellulose membranes for probing with troponin specific antibodies; for detection of troponin T we employed monoclonal antibody BN-59, whose preparation was described previously [19].

RESULTS AND DISCUSSION

The present results demonstrate that skeletal muscle troponin, and especially TnT, is substrate of transglutaminases. Unequivocal proofs of TnT modification by erythrocyte

transglutaminase are given by i) the calcium dependent formation of high MW aggregates which are stained by TnT monoclonal antibodies, ii) the incorporation of radioactive putrescine into TnT (and its aggregates), iii) the reaction of spermidine with troponin with the formation of isopetide bonds. During incubation with purified transglutaminase and high concentration of calcium ions, the aggregation state of skeletal troponins is heavily modified through generation of protein oligomers of apparent MW of 60 to 100 kDa and final production of high MW aggregates which do not migrate within low concentration polyacrylamide slab gels (Figure 1). The reaction is related to the activity of transglutaminase because aggregates resist boiling under reducing conditions and they are formed only in the presence of native enzyme and calcium ions, with inhibition by ammonium ions (a product of transglutaminase) and by GTP (an allosteric inhibitor) in the presence of suboptimal concentration of calcium [20]. Since the formation of γ-glutamylpolyamines is the unequivocal proof of transglutaminase catalysis [1], we also quantitated the amount of γ-glutamyl [^{3}H] spermidine formed between [^{3}H] spermidine and troponin as a result of transglutaminase action. The γ-amides produced in this reaction were released by exhaustive proteolysis, which hydrolyzed the peptide bonds leaving unaltered the much more stable isopeptide bonds of γ-glutamylpolyamines. As shown in Figure 2 there was a significative amount of [^{3}H] spermidine incorporated into troponin as either N^1- or N^8- (γ-glutamyl) spermidine (57% of the total radioactivity) and as N^1, N^8-bis (γ-glutamyl) spermidine (18% of the total radioactivity).

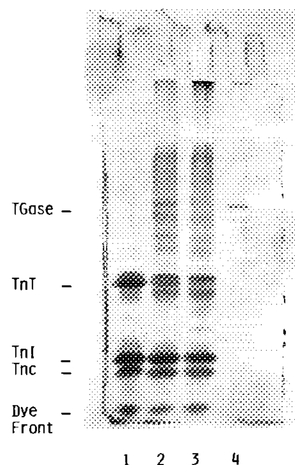

Figure 1. Polymerization of skeletal troponin as demonstrated by SDS-PAGE. Troponin was incubated with erythrocyte transglutaminase and 3 mM calcium acetate, as detailed in the Methods section. At timed intervals samples were withdrawn, boiled in the presence of 10 mM mercaptoethanol and 2% (w/v) SDS and electrophoresed on 10% polyacrylamide slab gels. Lane 1, purified troponin; lanes 2 and 3, troponin polymerized by incubation with transglutaminase and calcium ions for 30 and 90 minutes; lane 4, purified erythrocyte transglutaminase. Note the haevy precipitate formed in the wells in lanes 2 and 3.

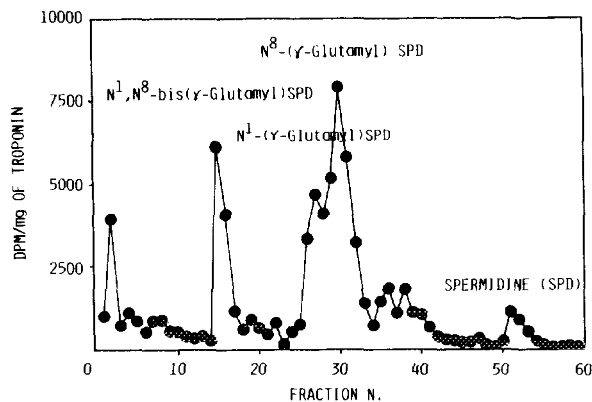

Figure 2. Identification of the transglutaminase-catalyzed products of troponin in the presence of [^{3}H] spermidine. Ion-exchange chromatographic separations of the proteolytic digest of modified troponin were performed as outlined in the Materials and Methods section. The identity of the three derivatives found was confirmed by the release of free [^{3}H] spermidine upon acid hydrolysis.

An alternative transglutaminase substrate, dansyl-cadaverine employed at 0.2 mM concentration, does not alter the polymerization state of transglutaminase-treated troponin, but leads to fluorescence labelling of the TnT subunit and of high MW aggregates (data not shown). In the troponin complex, TnT was clearly the most sensitive subunit for the transglutaminase catalyzed reaction since it disappeared rapidly in the stained slab gels concomitantly with the appearance of immunoreactive intermediates (Figure 3) and high MW aggregates. On the other hand other Tn subunits did not disappear in the gels (Figure 1) and aggregates were unreactive with anti-TnI and Tn-C antibodies (results not shown). Only TnT and its polymerization aggregates were significantly labelled following incubation with transglutaminase, calcium and radioactive putrescine, as demonstrated by autoradiography (Figure 3).

A minimal stoichiometry of the reaction was determined from the incorporation of radioactive putrescine into native troponin. Under these conditions we obtained incorporation of radioactivity up to 0.35 moles/mole of troponin, suggesting a probable 1:1 stoichiometry, taking into account the concomitant progress of the polymerization reaction. Thus only one, out of 9, glutamine residue in TnT subunit is recognized by type 2 erythrocyte transglutaminase.

The present results add TnT to the potential substrates that can be modified by transglutaminase in muscle tissues [8]. Especially in injured cells, the rise in $[Ca^{2+}]_c$ is likely to activate transglutaminase. The resulting cross-linking of troponin and other myofibrillar proteins could eliminate any residual probability of functional recovery. Indeed troponin aggregates have been observed (L. Gorza, unpublished results) in rat hearts subjected to either prolonged ischemia or calcium paradox [21]. In addition, cross-linked troponin molecules could represent an intracellular marker of irreversible damage with potential diagnostic relevance in the study of myocardial ischemia.

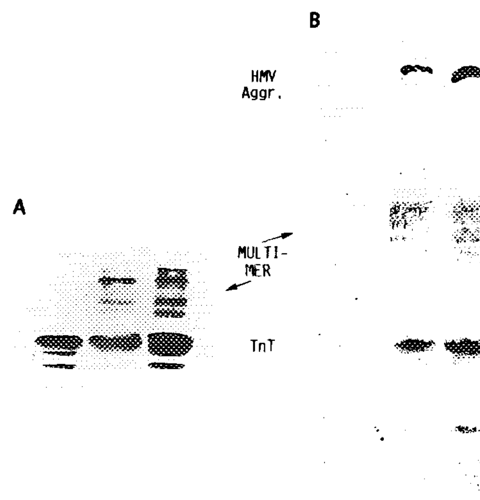

Figure 3. Identification of TnT as the preferred transglutaminase substrate in the Tn complex. A) reaction with antibodies specific for individual Tn subunits. Positive reaction was obtained only by employing antibodies against TnT, which stained TnT, the dimers and trimers, and the aggregates. B) Labelling of TnT and aggregated forms by incorporation of radioactive putrescine. The incubation was carried out as described in the Methods section.

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