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ORIGINAL ARTICLE

The side chain of glutamine 13 is the acyl-donor amino acid modified by type 2 transglutaminase in subunit T of the native rabbit skeletal muscle troponin complex

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Abstract Subunit T of the native muscle troponin complex is a recognised substrate of transglutaminase both in vitro and in situ with formation of isopeptide bonds. Using a proteomic approach, we have now determined the precise site of in vitro labelling of the protein. A preparation of troponin purified from ether powder from mixed rabbit skeletal muscles was employed as transglutaminase substrate. The only isoform TnT2F present in our preparation was recognised as acyl-substrate by human type 2 transglutaminase which specifically modified glutamine 13 in the N-terminal region. During the reaction, the troponin protein complex was polymerized. Results are discussed in relation to the structure of the troponin T subunit, in the light of the role of troponins in skeletal and cardiac muscle diseases, and to the rules governing glutamine side chain selection by tissue transglutaminase.

Keywords Transglutaminase · Skeletal troponin T · Protein post-translational modification

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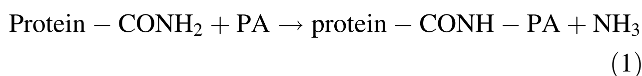
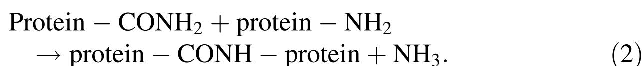
Introduction

Transglutaminases (Tgases) are enzymes which catalyse the modification of proteins at peptidyl-glutamine residues by a strictly calcium-dependent transfer of the acyl-moieties of the glutamine amido group to acceptor primary amines, with formation of isopeptide bonds. When the acyl-acceptors are constituted by soluble low-molecular weight amines, such as polyamines (PA), the reaction products are post-translationally modified proteins (protein transamidation). Alternatively, in the case the amine acceptor is the ε-aminogroup of protein-bound lysine residues, the Tgase reaction originates aggregates of polymerized proteins, which are frequently insoluble (protein crosslinkage) (Folk and Finlayson 1977).



$$\text{Protein} - \text{CONH}_2 + \text{PA} \rightarrow \text{protein} - \text{CONH} - \text{PA} + \text{NH}_3 \quad (1)$$



$$\text{Protein} - \text{CONH}_2 + \text{protein} - \text{NH}_2 \rightarrow \text{protein} - \text{CONH} - \text{protein} + \text{NH}_3. \quad (2)$$

The balance between the transamidation (1) and the crosslinkage (2) reaction catalysed by tissue transglutaminase (Tgase 2) is likely linked to the intracellular concentrations of PA (Lentini et al. 2007), which are the preferred amine substrates in transamidation. In this respect, we must remind that PA are considered "physiologic" substrates for transamidation (Folk et al. 1980) since the Km of Tgase 2 for polyamines falls in the cell physiologic range of polyamine concentration and PA-γ-glutamyl adducts are easily detectable in digested tissue extracts.

Tgase 2-mediated transamidation involves a few selected glutamine residues in substrate proteins (Esposito and Caputo 2005), because of strict rules of specific selection

not yet understood. Also the functional consequences of the type 2 Tgase-mediated protein transamidation are matter of discussion, since both enhancement and inhibition of the activity of modified proteins have been reported. Indeed, the original hypothesis that transglutaminases act as protein-modifying enzymes triggering physiologic responses by altering function in modified proteins (Esposito and Caputo 2005; Facchiano and Facchiano 2009) is now considered valid only in relation to the functions of Tgase 2 in the extracellular milieu. In contrast this "functional" effect is questionable in the intracellular space, as we discussed recently (Bergamini et al. 2011), since the transamidation reaction is irreversible. Tgase 2 is actually a cryptic enzyme under basic intracellular conditions, particularly because of effective inhibition by GTP coupled to the low concentrations of the essential activator calcium (Griffin et al. 2002). Under these conditions, the role of Tgase 2-dependent protein modifications is unlikely to affect protein/enzyme interconversion for metabolic regulation. These considerations seem to apply to all Tgase-mediated modifications at glutamine residues, including their hydrolysis to glutamic acid which is probably relevant in the pathogenesis of coeliac disease.

To further clarify the structural bases for the Tgase 2-mediated modification of peptidyl-glutamine residues, we explored the structural features around the site of labelling in proteins which are recognised Q-substrates for Tgases. In the present report we describe studies on skeletal muscle troponins, which we investigated in the past identifying the T subunit (TnT) as the preferred substrate of Tgase 2 in vitro and in situ (Bergamini et al. 1995; Gorza et al. 1996).

Materials and methods

Proteins

The troponin protein complex was purified from ethyl ether powders of rabbit skeletal muscle (Potter 1982) and Tgase 2 from human RBC (Gambetti et al. 2005) with the modification to incorporate a DTT-activation step before the final chromatographic separations. Sequence grade trypsin was obtained from Sigma-Aldrich.

Labelling of Tn subunits by Tgase 2

The reaction was carried out by incubating Tn complex (1–2 mg/ml) with purified Tgase in the presence of 5 mM calcium (and 1 mM spermine, when specified). Stoichiometry of glutamine labelling was checked in parallel incubations by measuring incorporation of radioactive putrescine into the troponin protein as previously described (Bergamini et al. 1995). To identify the glutamine residue whose side chain is modified by transglutaminase, incubations carried out in the presence of cold spermine were terminated by boiling in the presence of Laemmli denaturation buffer, and the troponin subunits were resolved by SDS-PAGE (Laemmli 1970). Relevant bands in gels stained according to (de Laurentiis et al. 2006), were excised from the gel, digested with trypsin and mass analysed by MALDI TOF and MALDI TOF–TOF techniques.

In-gel-digestion and MS identification of peptides

Coomassie blue-stained bands excised from the gel were washed in sequence with acetonitrile and with 50 mM ammonium bicarbonate pH 8.0. Protein samples in gel fragments were reduced by incubation in 10 mM dithiothreitol in ammonium bicarbonate for 45 min at 56°C and alkylated with 55 mM iodoacetamide in ammonium bicarbonate (30 min at room temperature in the dark) (de Laurentiis et al. 2006) and further washed with ammonium bicarbonate and acetonitrile. Enzymatic digestions were carried out with trypsin solution, 10 µg/ml, in 50 mM ammonium bicarbonate, pH 8.0, at 4°C for 1 h and further digested at 37°C for 18 h. A minimum reaction volume sufficient for complete rehydration of the gel was used. Released soluble peptides were acidified with 20% trifluoracetic acid (TFA), lyophilized, and resuspended in 10 µl of 2% TFA; 1 µl was loaded in the appropriate wells of the mass spectrometer plate. Peptides were mixed with 1 µl of matrix (α-cyano-4-hydroxyycinnamic acid 10 mg/ml) dissolved in a mixture of 70% acetonitrile and 30% TFA (0.2%).

MALDI-MS and MS/MS spectra were acquired using a 4800 plus MALDI TOF TOF mass spectrometer (ABI Sciex, USA). Protein identification was carried out by peptide mass fingerprinting using an in house version of the Mascot software. For troponins characterization, mass signals were mass mapped onto the anticipated sequences of rabbit skeletal muscle troponins as available in the Expasy site (entry P02641 for Troponin T).

Computational analysis

The sequence of Troponin subunits was numerated from the first amino acid of the coding sequence excluding the initial methionine present in the Swiss Prot data bank. Pairwise and multiple alignment of protein sequences was performed with CLUSTALW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) setting the BLOSUM protein weight matrix (Thompson et al. 1994). Disordered regions in protein sequences were predicted with DISOPRED2 (http://www.bioinf.cs.ucl.ac.uk/index.php?id=806) (Ward et al. 2004).

Results

Modification of troponins by Tgase

In these experiments, we proceeded with the identification of the reaction products of troponins following incubation with Tgase in the presence of spermine. The Tn complex was treated in vitro with purified Tgase in the presence of calcium ions and 1 mM spermine for 3 h at room temperature. The putative modified proteins were fractionated by SDS-PAGE. Figure 1 shows the corresponding gel stained with Coomassie blue, where the Tgase-modified troponins (lane M) were compared to a sample of unmodified troponins used as control (lane C).

Comparison of the two gel lanes showed a similar band patterns in the medium and low MW regions of the SDS-PAGE, whereas the troponins sample was specifically characterized by the occurrence of various bands in the high MW region that were absent in the control lane. Relevant bands in both the control and sample lanes were excised from the gel and submitted to the identification procedure as described in "Materials and methods".

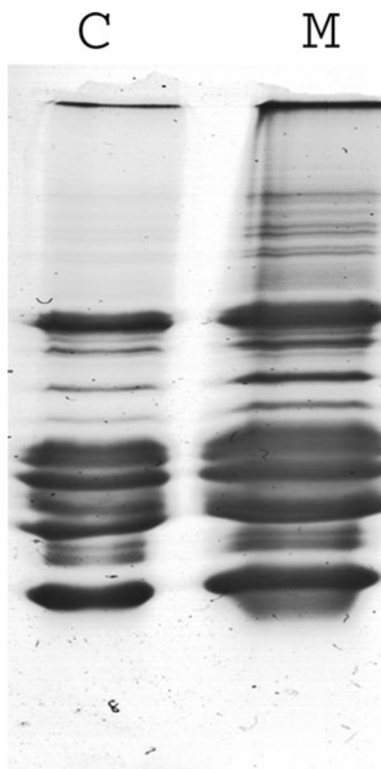


Fig. 1 Electrophoretic separation of troponin subunits by SDS-PAGE. The *lanes* labelled *C* and *M* refer, respectively, to a sample of control troponin and to a sample of troponin modified for 3 h by transglutaminase in the presence of calcium and spermine, under the conditions detailed in the "Materials and methods" section. The load of protein in the *lane M* was double than that in *lane C*

The peptide mass fingerprinting procedure led to the unambiguous identification of the proteins occurring in the three gel bands present in the medium–low MW regions of the gel. As reported in Fig. 1, the first band in this region corresponded to troponin T (Swiss Prot entry P02641) with the two other faster bands being troponin I (P02643) and troponin C (P02586), respectively. The pattern of protein identification obtained by MS analyses of the gel bands both in the control and sample lanes revealed that the troponin preparation contained the expected mixture of the three troponin subunits. Despite the theoretical possibility that multiple isoforms of the subunits are present as a consequence of the metabolic and functional heterogeneity of rabbit skeletal muscle (troponin T, the tropomyosin-binding subunit of troponin, in particular is reported to exist as nine isoforms produced by alternative splicing), the PMF procedure unambiguously identified the isoform present in the gel band as isoform 4 (also called isoform TnT2F).

Protein bands occurring in the high MW region were tentatively assigned to aggregates of troponin T generated by Tgase action. In addition to simple labelling of glutamine residues by incorporation of PA in the troponins monomer, it is well known that Tgase 2 also catalyses crosslinking of troponins into high MW polymers. These are likely stabilised by isopeptide bonds involving either directly lysine residues (Folk and Finnlayson 1977) or additional glutamine residues through a polyamine bridge to yield, under our conditions, aggregates stabilised by glutamyl-spermine-glutamine isopeptide bonds (Martinet et al. 1990). To confirm these assumptions, we tried to analyse peptides released by extensive trypsin proteolysis of the high MW bands. However, this approach was unsuccessful either because of the low amount of protein material or because of the resistance to proteolysis of the high MW aggregates. This is occasionally the case in protein aggregates generated by transglutaminase because the likely blockage of lysine residues and the tight assembly of the protein aggregates prevent efficient cleavage by trypsin (Ruoppolo et al. 2003). Conversely, it is unlikely that the crosslink involves a glutamine-PA-glutamine bridged isopeptide since sensitivity to proteolysis by trypsin should theoretically not be affected.

Identification of the specific Gln residues recognised by Tgase

Peptide mixtures from the tryptic digest of the protein bands corresponding to troponins in the Tgase-treated sample (lane M in Fig. 1) were carefully analysed to identify the Gln residues involved in transamidation in each individual troponin subunit.

Tryptic peptides from the gel band corresponding to troponin T were analysed by MALDI TOF and the corresponding mass signals mapped onto the anticipated sequence of the troponin T isoforms previously identified by PMF (entry P02641-4 in the Swiss Prot database). All mass signals recorded in the spectra could be assigned to predicted peptides from the troponin T sequence leading to the coverage of the entire protein primary structure. A single mass signal at m/z 5,247.6 could not be associated to any peptide within the troponin T sequence and was then tentatively interpreted as a Tgase-modified peptide.

The recorded mass value was 185.4 Da higher than the peak at 5,062.2 corresponding to the acetylated form of the N-terminal peptide 2-44, where Met 1 had been deleted. This mass difference could account for a spermine molecule covalently attached to the N-terminal peptide via an isopeptide bond catalysed by Tgase, with release of ammonia. Since this peptide contains a single Gln residue at position 13, this amino acid should be considered as the amino acceptor substrate of Tgase 2. However, this hypothesis had to be confirmed by tandem mass spectrometric analyses. The mass signal at m/z 5,247.6 was then isolated within the mass spectrometer and submitted to MS/MS analysis. Although the peptide mass value was particularly high, a very clear series of y ions could be detected in the daughter ion spectra, as shown in Fig. 2. Interpretation of the fragment ions led to confirm that this peptide indeed corresponded to the acetylated fragment 2-44. Moreover, the occurrence of the two key fragment ions at m/z 3,822.9 and 3,510.6 belonging to the y series clearly demonstrated that Gln13 was covalently modified by a spermine molecule via an isopeptide bond.

Similar analyses were carried out on the gel bands corresponding to troponin I and C from lane M of the gel depicted in Fig. 1. No altered peptides were detected whatsoever in the spectra of the tryptic digests corresponding to both isoforms where all the Gln residues were shown to be unmodified by Tgase. These data indicated that only troponin T is substrate of Tgase 2, with Gln13 at the N-terminus of the protein being the only labelled glutamine residue.

The structure of TnT around the site of labelling

The computational analysis of the region involved in labelling of the glutamine residue highlights some clues on the criteria for selection of glutamine residues by human Tgase 2. First of all, we compared the N-terminal sequences of the 3 subunits of skeletal troponin TnT, TnC and TnI. All these subunits include glutamine residues within the N-terminal sequence, as shown in Fig. 3a where the first 50 aminoacids of the three sequences are depicted. The most obvious peculiarity of the sequence of TnT around the labelled Gln 13 is the cluster of acidic residues (the sequence is rich in glutamic acid residues). This feature is not present in the other Tn subunits (TnI and TnC), while it is maintained also in other variants of TnT which are generated by alternative gene splicing (Fig. 3b). In these instances, the sequence preceeding Gln 13 (MSDEEVEHVEEEAQ) of the TnT2F variant is converted into the sequence MSDEEVEHVEEQYEEEEEAQ due to the expression of a glutamine containing peptide EEQYEE. On the basis that local information as derived from the primary structure is relevant for the recognition of TnT variants, the glutamine recognition should occur also in the other variants which conserve the same sequence motive around the conserved glutamine (Fig. 3b). We do not have this information yet because only the variant TnT2F was present in our preparation of troponins from rabbit fast skeletal muscle. Notably in human Annexin 1 which is a recognised transglutaminase substrate, Gln 19 is also surrounded by glutamic acid residues. It has been demonstrated in the pig counterpart, which shares a 88.7% sequence identity with human Annexin 1, that an identical Gln 19 flanked by glutamic acid residues is present in the N-terminal region. This region is disordered in the presence of calcium, but it undergoes folding to a flexible α helix in the absence of the cation (Rosengarth et al. 2001; Rosengarth and Luecke 2003).

To investigate additional features of TnT2F that might participate in glutamine recognition, we have considered secondary structure and local flexibility. Since these parameters are not directly available from studies in the crystalline state, we have taken advantage of predictors to gain this information. According to our data (Fig. 3a, where disordered residues are shown in italics), Gln 13 is predicted to be located in a long disordered region. This region should be then characterized by an appreciable flexibility, since the analysis of segmental "rumor" corroborates the notion that both substrate regions in TnT2F and human Annexin I are very disordered. In turn, the N-terminal regions of TnI and TnC are characterized by considerable rigidity. The analogous regions in the other skeletal muscle TnT variants which are presumed to be potential substrates are characterized by analogously high flexibility (data not shown).

Discussion

Gard and Lazarides (1979) described labelling of structural proteins of the Z-line in glycerinated chicken myofibrils by Tgase 2 through incorporation of the fluorescent probe Dansylcadaverine in the presence of calcium. The authors identified α-actinin, tropomyosin and actin as the labelled proteins. Labelling of actin was later employed to

Fig. 2 Pattern of transamidation of glutamine residues in subunit T of skeletal troponin. The band corresponding to the monomeric subunit T of troponin was excised from the gel in Fig. 1 and processed for fragmentation MS as detailed in the "Materials and methods" section. The sequence identified in the only modified peptide with m/z 5,247.6 is reported in this picture starting from the top-right corner. The missing glutamine (residue Q13) is marked by *asterisk*

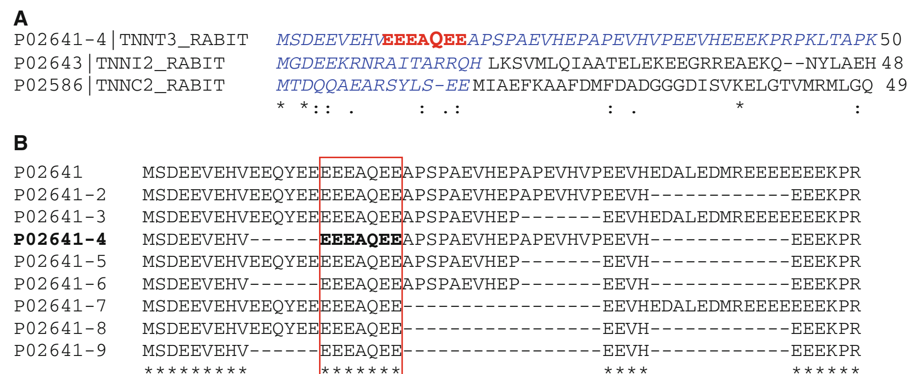


Fig. 3 **a** Sequence alignment of the amino-terminal region of the rabbit Tn subunits TnT, TnI and TnC. The sequence labelled in the TnT subunit is marked in *bold*, while regions of elevated disorder are presented in *italics*. **b** Comparison of the sequence of the known isoforrns of the rabbit TnT subunit to document the conservation of the labelled region in TnT2F also in the other variants

investigate actin polymerization which proceeds unaltered, since the label does not grossly interfere with the functional properties of actin itself provided that an intramolecular crosslink between Q41 and K50 is not formed (Eli-Berchoer et al. 2000). At the same time, we investigated labelling of troponins by Tgase 2 in the perfused heart after calcium stress (Gorza et al. 1996), and in vitro employing skeletal muscle troponin as substrate (Bergamini et al. 1995). In our hands, troponin T was the main site of labelling. At variance with our results,

McDonough et al. (1999) reported that also TnI is a substrate for Tgase 2 and that it is crosslinked to the C-terminal (190-275) proteolytic fragment of TnT into aggregates which are released in heart perfusates following infarction/reperfusion injury. Such discrepancies prompted us to clarify what is the precise site of labelling and what factors can play a role about, since troponins are clinically relevant markers of muscular and cardiac cell dysfunction (Panteghini 2009).

The present findings confirm and extend our previous observations since modification of troponin occurs only at subunit T, labelling glutamine 13 at the N-terminus of the single isoform present in the protein preparation we have used. Identification of this glutamine Q13 as the site of labelling was achieved by analysing products of incubations carried out in the presence of exogenous polyamines. This glutamine is flanked by acidic residues (which might mitigate the effects of the positive charges brought about by labelling by Tgase in the case polyamines act as acyl-acceptors) at the end of a loose and flexible α-helix. As mentioned in the text, this same motive is present and substrate of Tgase 2 in human Annexin 1 (Pepinsky et al. 1989) can undergo reversible shifts between a random coil and a mobile flexible α-helix in porcine Annexin 1 (Rosengarth and Luecke 2003). These considerations are consistent with hypotheses that either sequence-charge or mobility effects are relevant for glutamine recognition (see also below). In contrast to the definitive identification of Glutamine 13 as the acyl-donor aminoacid, we could not reach any conclusion on the identity of acyl-acceptor in the crosslinked products because of the marked resistance of the polymer to proteolysis, which might be due to an intrinsic resistance of the polymer to degradation or of the inability of trypsin to digest aggregated proteins whose lysine residues are blocked in isopeptide bonds, as outlined in the "Results" section. We will investigate further this issue by means of additional proteinases of different specificity (e.g. the staphylococcal V8 protease) or of artificial glutamine acyl-donors such as CBZ-glutaminylglycine, which is an usual peptidyl-glutamyl substrate of Tgase 2. This last approach was useful in previous studies on the identification of substrate lysine residues using polyglutamine tails in other systems (Ruoppolo et al. 2003).

An issue deserving consideration is related to the specificity of glutamine labelling which might arise from (i) sequence and 3D structure around the labelled glutamine and from (ii) possible variation of substrate activity in relation to additional protein post-translational modifications. We must remind that in this respect controversial data have been published. In the case substrates are libraries of short random peptides containing Q residues (Sugimura et al. 2006), issues based on primary structure motives are probably relevant since preferential labelling of glutamines occurs at residues flanked by hydrophobic residues on the C-terminal side. In these studies, however, contributions arising from the limited folding of these short (decameric) peptides have been disregarded. In contrast, combined data obtained on intact proteins behaving as glutaminyl-substrates (Esposito and Caputo 2005), further summarised in the TRANSDAB database available at the University of Debrecen (Csosz et al. 2009), indicate that labelling takes place independently of primary structure, but are rather linked to the 3D arrangement, at variance with what happens with other protein modifying enzymes, e.g. the protein kinases (Pinna and Ruzzene 1996) which recognise sites of specific labelling from the primary structure of the protein substrate. The 3D structure is probably the predominant factor in dictating substrate specificity of mammalian transglutaminases since for instance labelling of fibronectin by Tgase 2 and by Factor XIII occurred at multiple different sites and was increased by limited proteolysis of the substrate, likely because of exposition of additional sites hindered in the native protein or of conformational changes (Fesus et al. 1986). These effects, which might associate with focal loss of compact structure, might explain the discrepancies between these and our previous in vitro findings (Bergamini et al. 1995) and those reported by McDonough et al. (1999) in the perfused heart under ischemic conditions, since in this last model activation of proteolytic cascades and nicking of substrate proteins likely occur. It is thus apparent that sequence issues are relevant determinants of glutamine-specific recognition only in short unstructured peptides but not in large proteins in which protein secondary and tertiary structures are important for selectivity.

Data obtained with bacterial transglutaminase support this view. For instance, it has been proved (Mero et al. 2009) that bacterial Tgase is selective in modifying only one (Q134) out of the 17 glutamine residues within the 174 amino acids sequence of granulocyte-CSF. According to these authors, the most relevant parameter in Q-selectivity is represented by protein segmental flexibility around the substrate glutamine (estimated from a high value of crystallographic B-Factor), which dictates fitting of the substrate region in the enzyme active site, as it happens in the case of proteinases. In our instance, the glutamine residue acted upon by human transglutaminase in TnT4 is harboured in a region of high flexibility, as it happens for a mutant of yeast phosphoglycerate kinase (Coussons et al. 1991).

Other interesting implications might be related to the fact that only troponin T is acted upon by Tgase 2. This subunit is crucial in dictating the functional properties of the calcium-dependent ATPase activity of myosin. Indeed, the functional differentiation of muscle in fast- and slow-contacting forms is accompanied by shifts in TnT expression, which also occur during development in skeletal and

in cardiac muscles (Chaudhuri et al. 2005; Feng and Jin 2010). As mentioned, the TnT isoforms have different calcium sensitivity, and chiefly differ for their N-terminal regions (Wang and Jin 1998), actually that susceptible to the Tgase-mediated modification. If we also take into account the associations between over-expression of Tgase 2 and susceptibility to cardiac failure in transgenic animals (Small et al. 1999) as well as the presence at high title of anti-Tgase 2 antibodies in serum of patients with end-stage cardiac failure (Peracchi et al. 2002), it is possible that different tissular contents of expressed TnT isoforms with different reactivity towards Tgase-mediated transamidation, might be relevant in clinical pathology. Clearly, further experiments are required to verify these intriguing hypotheses, at the light of their possible clinical relevance and the three-dimensional assembly of the subunits of troponin complex, which is the object of intensive investigation in several laboratories (Kowlessur and Tobacman 2010).

Conflict of interest The authors declare that they have no conflict of interest.

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