“Tissue” transglutaminase contributes to the formation of disulphide bridges in proteins of mitochondrial respiratory complexes

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Abstract

In this study we provide the first in vivo evidences showing that, under physiological conditions, “tissue” transglutaminase (TG2) might acts as a protein disulphide isomerase (PDI) and through this activity contributes to the correct assembly of the respiratory chain complexes. Mice lacking TG2 exhibit mitochondrial energy production impairment, evidenced by decreased ATP levels after physical challenge. This defect is phenotypically reflected in a dramatic decrease of motor behaviour of the animals. We propose that the molecular mechanism, underlying such a phenotype, resides in a defective disulphide bonds formation in ATP synthase (complex V), NADH-ubiquinone oxidoreductase (complex I), succinate-ubiquinone oxidoreductase (complex II) and cytochrome c oxidase (complex IV). In addition, TG2-PDI might control the respiratory chain by modulating the formation of the prohibitin complexes. These data elucidate a new pathway that directly links the TG2-PDI enzymatic activity with the regulation of mitochondrial respiratory chain function.

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1. Introduction

Transglutaminases (TGases) are a family of calcium dependent cross-linking enzymes involved in post-translational modification of proteins. TGases catalyse intra- and intermolecular isodipeptide bonds, between the γ-carboxiamide groups of glutamine residues and primary amino groups of several compounds. Different isoforms with diverse tissue distribution are known [1,2]. The most ubiquitous isoenzyme is represented by type 2 transglutaminase (TG2), a versatile multifunctional protein involved in a variety of biochemical functions. In the presence of high calcium levels, TG2 catalyses protein–protein cross-linking as well as incorporation of primary amine into proteins [3]. It is well known that TG2 possesses other functions than the Ca2+-dependent cross-linking activity; in fact, it can act as a G-protein, coupling adrenoreceptors, thromboxane and oxytocine receptors to phospholipase C (PLCδ1). This activity is inhibited by Ca2+, which acts as a switch between the two enzyme’s main functions [4]. Recently, on the basis of in vitro observations, a novel Ca2+-independent disulphide isomerase activity has been hypothesized for the enzyme [5]. In the past years, TG2 has been widely related to programmed cell death, being selectively expressed during apoptosis [6]. Its ectopic overexpression increases the levels of apoptosis in various cell lines, conversely, its silencing leads to a decrease in cell death levels [7]. The deregulation of enzyme activity, generally is associated with major disruptions in cellular/tissue homeostasis and results in the enzyme contributing to a number of human diseases such as coeliac disease, neurodegeneration, maturity onset diabetes and tissue fibrosis/scarring [3]. TG2 gene deletion as well as chemical inhibition of its enzymatic activity improves symptoms as well as life spanning in Huntington’s disease.
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2. Materials and methods

All reagents, unless otherwise indicated, were purchased from SIGMA.

2.1. Animals

All animals were housed and farmed according to the guidelines proposed by the Italian National Research Council. Animals were sacrificed through cervical dislocation.

2.2. Antibodies

Antibodies against respiratory complexes proteins were purchased from Molecular Probes. The used dilutions were respectively: complex I 39 kDa subunit and 17 kDa subunit 0.5 μg/ml; complex II 70 kDa subunit 0.1 μg/ml and core 2 0.01 μg/ml; complex III core 1 (51.6 kDa) 0.1 μg/ml and core 2 (48.5 kDa) 0.4 μg/ml; complex IV 57 kDa subunit 2 μg/ml and 20 kDa subunits 0.2 μg/ml; complex V subunit α (59.8 kDa) 0.5 μg/ml and subunit β (56.6 kDa) 0.2 μg/ml. Anti-prohibitin antibody was purchased from NeoMarkers and dilution used was 0.2 μg/ml. Anti-Hsp60 antibody was purchased from Stressgen and dilution used was 1:10000.

2.3. Identification of TG2 substrates

TG2 over-expressing cells (TGA) were grown as previously described [13] in the presence of 5 mM TGase labelling substrate 5-(biotinamido)pentylamine (EZ-link, Pierce) and 1 μM staurosporine (STS) for 3 h.

2.4. Behavioural assay

Spontaneous locomotor activity was measured in a computer-controlled apparatus consisting of eight toggle-floor boxes, each one divided into two compartments (20 x 10 cm) connected by a 3 x 3 cm opening, as previously described [15]. For each mouse the number of crossings from one compartment to the other, by means of a micro-switch connected to the tilting floor of the box, was recorded for 60 min. Scores of consecutive 10-min periods were calculated. The apparatus was located in a sound-insulated cubicle. All values are expressed as means ± SEM.

2.5. Blue native electrophoresis

Complexes were solubilised according to a modified protocol of Schagger and von Jagow [16]. Briefly, mitochondrial pellet was suspended in 8 volumes of buffer (750 mM amionacric acid, 50 mM Bis-Tris, protease inhibitor cocktail (Roche) and 1 volume of 10% laurylmaltoside. The mixture was subsequently centrifuged at 100000 g for 15 min. The supernatant contains solubilised mitochondrial complexes. Blue Native electrophoresis was performed on a Protean II xi cell (Bio-Rad). The 5%–13% polyacrylamide gradient gels in amionacric acid were prepared as described. Electrophoresis was led at 4 °C and 75 V until proteins entered the stacking gel and, then, the cathode buffer containing G250 Coomassie Blue (Serva) was replaced by a buffer without the dye. Electrophoresis was continued over night at 150 V. Gels were stained with G250 Coomassie over night.

2.6. Western blot analysis

Tissues were collected immediately after sacrifice and homogenised in sucrose 250 mM, Tris–HCl pH 7.4 20 mM, EDTA 1 mM and protease inhibitors (Complete, EDTA free, ROCHE). Mitochondria were isolated as previously described [17]. Proteins were quantified with standard Bradford staining. 20 μg of proteins were separated on 5–12% Nu-PAGE (Invitrogen) and transferred to nitrocellulose membrane. Membranes were probed with the above-described antibodies and the signals were detected with Supersignal (Pierce).

2.7. Measurement of ATP levels

Adenosine 5′-triphosphate (ATP) levels were measured on liver, brain and skeletal muscle from wild type and knock out mice, by means of the ATP Bioluminescent Assay Kit (Sigma), according to manufacturer’s indications. Each sample was mixed with 100 μl of reaction buffer, vortexed briefly, and light output was measured immediately in a luminometer (Tuner TD). A standard curve ranging from 10^−14 to 10^−7 moles of ATP has been performed prior to each experiment.

2.8. Statistical analysis

All the data reported are the mean of at least four different experiments. Using Student’s t-test performed statistic analysis, only p-values of less than 0.01 were considered as significant.

3. Results

3.1. TG2 knockout mice exhibit mitochondrial defects

We have previously shown that TG2 protein levels correlate with alterations of mitochondrial membrane potential (ΔΨ) and
that TG2 over-expression causes a mitochondria hyperpolarization. This effect might be due to enhanced proton pumping by mitochondrial respiratory complexes and, interestingly, is present not only in cells undergoing apoptosis, when TG2 activity drastically increases, but also in steady state physiological conditions, when TG2 activity as well as Ca\(^{2+}\) levels are rather low [13]. We hypothesize that, apart its complex role in cell death [3,18], in physiological conditions TG2 might participate to mitochondrial homeostasis. Moreover, these evidences suggested to us that the absence of TG2 might results in alterations of the respiratory chains that should have consequences on the global production of ATP in tissues. If this is the case, such a difference should be more evident and highlighted, by a prolonged physical challenge, in those organs, which are not predominantly glycolytic, while should be not relevant in resting conditions, where ATP reserves are not depleted. To verify this hypothesis, we compared ATP content in WT versus TG2\(^{-/-}\) mice in resting condition as well as after a prolonged physical activity. In particular, we analysed the liver, whose metabolism widely relies on glycolysis and the skeletal and the cardiac muscles, where mitochondrial phosphorylative oxidation is, mainly, in charge of ATP production. We failed to detect any major difference in all tissues analysed under resting condition (data not shown). On the contrary, after prolonged physical effort (mice were kept running on Rota Rod at constant low speed and after 10 min the speed was progressively increased until the mice fall down), the ATP content in skeletal and cardiac muscle from TG2\(^{-/-}\) mice was drastically depleted. In particular, in the heart, where an utmost severity was found, we observed about 40% of ATP loss, when compared to controls (Fig. 1a–c).

These findings support the hypothesis that TG2\(^{-/-}\) animals should exhibit a phenotype as a direct consequence of mitochondrial defects. To verify this hypothesis we monitored the animal’s general activity and indeed we found that the spontaneous locomotor activity was significantly lowered in KO versus WT mice. ANOVA analysis of repeated measures demonstrated a significant main effect for genotype (\(F(1,85)=10.495;\ p<0.01\)), time (\(F(5,85)=68.318;\ p<0.001\)) and genotype×time interaction (\(F(5,85)=5.240;\ p<0.001\)) (Fig. 1d).

### 3.2. BN-PAGE does not reveal differences in TG2 knockout animals

On the basis of these evidences we decided to analyse the mitochondrial respiratory complexes (OXPHOS) in TG2\(^{-/-}\) animals by means of Blue Native electrophoresis, a technique that allows to separate complexes from mitochondria in their native state [16]. Fig. 2a shows, considering the detection limits of the technique, minor differences in the electrophoretic properties of complexes II, III and V between TG2\(^{-/-}\) mice and

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![Fig. 1. Measurement of ATP levels in tissues from wild type and TG2\(^{-/-}\) mice after prolonged physical challenge (a, b, c). ATP levels are comparable in the liver (a), a tissue with pronounced glycolytic activity, while major differences are detectable in skeletal muscle (b) and heart (c), where ATP production is mainly due to mitochondrial activity. TG2\(^{-/-}\) animals exhibit a reduced general activity and motility (d). Monitoring of the mice motility shows that after 50 min, wild type mice are still active while TG2\(^{-/-}\) animals are definitively resting.](image-url)
the respective controls. In order to analyse variations, in terms of protein composition, in each single complex, we performed a second dimension electrophoresis in reducing conditions (Fig. 3b) but also this approach does not reveal any major appreciable difference. We concluded that, although the level and the quality of the respiratory complexes are not significantly affected, TG2 gene ablation might result in some changes at the post-translational level.

3.3. TG2 acts as a disulphide isomerase in vivo

TG2 is a multifunctional enzyme and intracellular levels of calcium and GTP strictly regulate its cross-linking activity. Our experimental settings, being merely a physiological state, does not allow the activation of the cross-linking activity of TG2, so we decided to investigate possible alternative molecular mechanisms underlying our findings. A recent report, based exclusively on in vitro evidences, suggests that, in the presence of low calcium levels, purified TG2 is able to catalyse the formation of disulphide bonds [5]. In order to evaluate whether TG2 might function as a disulphide isomerase in vivo we analysed the formation of disulphides in mitochondrial respiratory complexes by means of non-reducing SDS-electrophoresis of the BN-PAGE. Fig. 2c shows how some of the high molecular weight bands present in complexes belonging to wild-type mice mitochondria disappear in TG2−/− ones. On the contrary, the pattern of bands under reducing conditions is identical (compare Fig. 2b and c). These findings were also
confirmed by normalisation of the reported gels (Fig. 2b and c) by densitometric analysis. Being β-mercaptoethanol the only variable introduced here, it is reasonable to think that the absence of disulphide bonds is responsible for the detected variations. In keeping with the above reported data, demonstrating that functionality of complex V and ATP synthesis is impaired in TG2−/− mice, we show that this complex as well as other complexes (I and III) have major variations in disulphide bonds when TG2 is depleted. In keeping with these findings, we measured complex I activity on heart mitochondria isolated from wild type and knock out mice. We found a 30–35% reduction (5.5±0.18 nmol NADHox/mg protein wt vs. 3.93±0.49 nmol NADHox/mg protein KO) in the activity of complex I NADH-ubiquinone oxidoreductase in heart's mitochondria from TG2−/− mice as compared to WT mice.

3.4. Identification of TG2 substrates in mitochondria

Although TG2 is considered mainly a cytosolic protein, there are several evidences showing that the enzyme may also display a mitochondrial localization [13,19]. In addition, even TG2 substrates co-localize with mitochondria [14], suggesting that lack of TG2-mediated post-translational modification in some mitochondrial proteins could determine the mitochondrial defect previously described in TG2 knock-out mice.

In order to identify which proteins are substrates of the TG2/ PDI activity we analysed a number of mitochondrial respiratory chain complex components as well as chaperone proteins known to play a role in mitochondrial OXPHOS protein import and assembly [20–22]. We recently demonstrated that Hsp60 and prohibitin act as substrates for TG2 cross-linking activity [14]. We performed western blot analyses of proteins belonging to mitochondria isolated from both wild type and TG2−/− mice tissues, under reducing and non-reducing conditions. Under reducing conditions, as far as prohibitin is concerned, the levels of the monomeric protein are comparable in both TG2−/− and wild type mice (Fig. 3a, lanes 2, 4). Interestingly, under non-reducing conditions, when disulphide bonds are preserved, mitochondria from mice lacking TG2 exhibit a drastic reduction in the levels of the high molecular weight products (Fig. 3a, lanes 1,3), clearly indicating a direct in vivo correlation between disulphide bonds formation and the presence of TG2. Moreover, in wild type animals, the amount of the 30 kDa band, corresponding to the monomeric form of the protein, is lower under non reducing as compared to reducing conditions (compare lanes 3 and 4). These observations suggest that in the heart, in a physiological context, a relevant part of prohibitin is present in high molecular weight complexes assembled through TG2-mediated disulphide bonds. Conversely, the other chaperone analysed, Hsp60, is not modified by the TG2 isomerase activity (Fig. 3b).

Prompted by these results, we then investigated whether other intrinsic protein components of the respiratory complexes are modified by the TG2/PDI activity. Protein from mitochondria belonging to WT and TG2−/− mice, were analysed by means of antibodies directed against 10 different components of the five respiratory chain complexes. As shown in Fig. 4, some of them are largely modified in TG2−/− mice, whereas others are not affected, thus indicating a high specificity in the TG2/PDI action. It is interesting to note the difference in the assembly of the ATP-synthase β-subunit, which is an important component of the complex V, which is responsible for the ATP synthesis.

To further address whether being a TG2 substrate for the isomerase activity implies the necessity of being a substrate for the cross-linking one, we investigated whether proteins, which show TG2 dependent modification in disulphides, are also substrates for the cross-linking activity. To this aim, we used TG2 over-expressing neuroblastoma cells, undergoing apoptosis in response to staurosporine treatment, coupled to the labelling of TG2 protein substrates with a biotinylated pentlyamine (EZ-Link) [23]. This compounds enters living cells and acts as a tag, being cross-linked to glutamine donor substrates by TG2, allowing purification of labelled substrates [24]. Purified mitochondrial protein substrates were analysed by western blot with streptavidin-HRP and specific antibodies for those proteins, which exhibit variations in disulphide contents. As shown in Fig. 5, although both the analysed proteins are substrates for the isomerase activity, the 39 kDa subunit of complex I is not a substrate for the cross-linking reaction. These findings, together with the evidence about HSP60 [14], which is, vice versa, substrate for the cross-linking activity but not for the isomerase one, indicate that these two enzymatic activities are unrelated.

4. Discussion

The data reported in this study provide the first in vivo evidence for the existence of a novel TG2 activity, namely Protein Disulphide Isomerase, and showed that through this
activity the enzyme participates to mitochondrial respiratory functions. In fact, we observed that disulphide bonds content of mitochondrial respiratory complexes is altered in mice lacking TG2. This phenomenon leads to a failure in the ATP production, which is phenotypically reflected in a dramatic decrease of general motility of these animals. Although a protein disulphide isomerase activity was reported to be associated with a cross-linking activity in an ERp60-like protein from the filarial parasite *D. immitis* [25] and this evidence was confirmed *in vitro* by using purified mammalian TG2 [5], no proof of the existence of such an activity as well as its relevance under physiological settings in vertebrates *in vivo* was reported so far for any TGases. Our data indicate that TG2 plays a physiological role as part of the folding machinery that controls the assembly of the respiratory complexes in mitochondria, in particular under stressful conditions. We demonstrated that the structure of some of the essential elements (see later) of the complex I, II and V are affected in TG2−/− mice. This study was prompted by our
previous observation showing that a significant fraction of the total cellular TG2 is localized on mitochondria, where it may affect both the ultra-structural and redox features of the organelle [13]. We have recently characterized prohibitin, HSP60 and ATP synthase beta chain as mitochondrial TG2 protein substrates, by using a neural cell line constitutively over-expressing the enzyme [14]. Interestingly, these two proteins are widely involved in the correct assembly and function of the mitochondrial proteins, with particular regard to the respiratory chains protein components [21]. In particular, prohibitin acts as a membrane bound chaperone and it has been shown to be essential for the correct folding of the mitochondrial membrane proteins [21]. HSP60, together with its essential partner protein mtHsp70, forms a membrane-associated import motor complex responsible for vectorial polypeptide movement and unfolding of pre-protein domains. For some substrate proteins, the protected folding environment, provided by the large oligomeric Hsp60 complex, further facilitates the folding reactions [22]. In addition we detected that, under non-reducing conditions, the electrophoretic features of a major component of the complex V, the ATP synthase beta chain, results to be modified in absence of TG2. Although this finding might seem misleading, since the ATP synthase beta chain does not have any cysteine residue, it must be noticed that a number of reports show that this protein behaves as it has cysteine in its primary sequence and has been identified in various instances as a cysteine containing enzyme [26].

TG2 is a widely distributed peculiar enzyme that catalyses the post-translational modification of proteins by the formation of isopeptide bonds. This may occur through protein–protein cross-linking, via epsilon-(gamma-glutamyl)lysine bonds, or through incorporation of primary amines in peptide-bound glutamine residues. The cross-linked proteins are oligomeric products, highly resistant to mechanical challenge and proteolytic degradation and their accumulation is specifically found in a number of pathological settings associated with cellular stress and/or death. The Ca$^{2+}$ is the essential activator of this transamidating activity, with the main binding site located at the terminal $\alpha$-helix (H4) in domain 2 [2]. Although some potential regulatory mechanisms (interaction of TG2 with lysophosphatidylcholine, nitrosylation of the active site cysteine 277) suggest how the enzyme might acquires some activity at near physiological levels of Ca$^{2+}$, up to now, it is generally assumed that under physiological conditions TG2 is largely inactive as cross-linking enzyme. In keeping with this assumption, we did not find any oligomerization of the putative mitochondrial protein partners in living cells and only at a very little extent in dying ones. Interestingly, TG2/PDI folding activity does not require high calcium levels and then may occur under steady state physiological conditions [5]. By studying the status of several essential components of the respiratory chain complexes, under native versus reducing conditions, we found that the observed phenotype for TG2$^{-/-}$ mice might be probably due to an improper post-translational modification of the 17 kDa and 39 kDa subunits of succinate–ubiquinone oxido-reductase (complex I), the 30 kDa and 70 kDa subunit of succinate–ubiquinone oxido-reductase (complex II) and 20 kDa subunit of cytochrome c oxidase (complex IV). In fact, western blot analysis of these proteins under non-reducing conditions, revealed an evident impairment in the extent of TG2-mediated disulphide bond formation. The TG2/PDI activity appears to be very specific, as clearly indicated by the fact that only a fraction of the analysed proteins were affected in TG2$^{-/-}$ mice. In particular, the reduced efficiency in ATP synthesis is associated with lack of disulphide bonds only on the beta-chain of ATP synthase. Defects observed in TG2$^{-/-}$ mice could be due both to a direct action of TG2 on the reported proteins as well as to the absence of TG2-catalysed post-translational modification of the important chaperone prohibitin. Prohibitins, in eukaryotes, consist of two subunits that form a high molecular weight...
complex localised in the inner mitochondrial membrane [21]. The evolutionary conservation and the ubiquitous expression in mammalian tissues of the prohibitin complex suggest an important function among eukaryotes and the high molecular weight prohibitin complex has been shown to play an essential role in the stabilization of newly synthesized subunits of mitochondrial respiratory enzymes [27].

The observed defects in the correct assembly of mitochondrial respiratory complexes result in a reduced ability to synthesize ATP (particularly under stressful conditions) and a consequent adult phenotype in TG2−/− mice. Although these mice do not exhibit major embryonic anomalies, it is important to note that the energetic supplies during embryonic life are largely provided by maternal tissues, as demonstrated by the fact that, even in the most severe human mitochondrial diseases, death generally occurs in early post-natal life. However, adult TG2−/− mice show profound differences in behaviour, reflected in a drastic reduced motorial activity. Moreover, these animals showed a profound ATP debt, following physical challenge, mainly in non glycolytic tissues. Interestingly, even the defect in insulin secretion (MODY) previously observed in these animals could be, at least in part, explained by the mitochondrial assembly defects described here, being this pathology also related to ATP defects [12]. In line with this consideration the TG2−/− mice represent a very interesting model to study human pathologies characterized by a defect in the assembly of mitochondrial protein complexes.

In conclusion, despite extensive investigations, the questions of the physiological role played by TG2 and the relevance of the post-translational modifications of its protein substrates are open questions. In this study we present compelling evidences, indicating that the enzyme may act as a Protein Disulphide Isomerase. The physiological PDI activity described in this study is well complemented by the transamiditating TG2 activity. Under stressful circumstances, such as those induced by many cell death stimuli, the increase of intracellular calcium concentration switches on TG2 cross-linking activity and determines the active involvement of the enzyme in apoptosis as previously reported [3]. Thus, TG2 represents another example of a cell death-associated protein able to play different functions according to different cellular context and needs [28]. In addition this study demonstrated, in vivo, the importance of disulphide bonds formation in the correct assembly of the mitochondrial respiratory chain complexes and consequently in the energy production pathways in the eukaryotic cell.

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References


