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EDITORIAL

Transglutaminase 2, a double face enzyme

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Introduction

During the years *Amino Acids* have dedicated much attention to the transglutaminase (Tgase) field and this special issue is therefore the fifth of a series. In a previous occasion, we celebrated the 50 years from transglutaminase discovery with the editorial "An overview of the first 50 years of transglutaminase research" (Beninati et al. 2009). Following ideally from that point, we have now chosen as general theme for this issue the multiple roles played by type 2 transglutaminase as a multi-face protein.

Most people are accustomed to analyze events and facts by taking into account objects and their opposite to get the whole, as in the case of light and darkness, day and night, or black and white as within a chessboard. This is also the case for type 2 transglutaminase (Tgase2): to get the complete picture we must examine both sides of the problem, the protein with its opposite activities, the involvement in cells and tissues leading either to cell growth/differentiation or conversely to cell death/atrophy, and their implications in health protection and pathology. The aim in launching this special issue was to contribute to settle these topics and we introduce now these general concepts dividing ideally the path in the steps mentioned above.

The physiology: the enzyme and its multiple activities

Researchers who joined the transglutaminase field long ago as we did witnessed the huge progression in Tgase research since the time it represented a niche issue, mainly a curious enzyme without any evident biologic function. The research focused mainly on the enzymatic properties and the nature of the catalyzed reaction, while the possible physiologic functions as well as the relevance in pathology were just matter of sheer speculation at the end of the discussion in our papers. This scenario changed rapidly as different isoenzymes were recognized initially based on the thermal stability and on different responses to treatment with retinoids (Lichti et al. 1985), and finally proved with cloning and sequencing (Gentile et al. 1991) and definite recognition of different isoenzymes. In any case, Tgases were still considered mainly as protein crosslinking enzymes and the ingenious filter paper assay technique developed by Laszlo Lorand (1972) remained fundamentally a useful experimental tool at least independently of the function of polyamines as endogenous substrates for the reaction as proved later in Jack Folk's lab (Folk et al. 1980). Two other seminal discoveries changed the landscape, the notion that the activity of Tgase (again Tgase2) was linked both to cell growth and programmed death (Haddox and Russell 1981; Fesus et al. 1987) and that it is subjected to inhibition by GTP (Achyuthan and Greenberg 1987; Bergamini et al. 2010). To complete the picture, it was demonstrated shortly thereafter that Tgase2 can bind to membranes acting as transducer of external endocrine messages

This work is dedicated to the memory of Prof. Alberto Abbruzzese, who died in 2011.

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likely as a G-protein (Nakaoka et al. 1994). It emerged that the transamidating and GTP-ase activities of Tgase2 are closely regulated by the availability of calcium and GTP so that the transamidation activity is probably largely inactive in the intracellular compartment as originally assumed and checked recently in studies on the conformation of the enzyme inside the cells (Pavlyukov et al. 2012; Diaz-Hidalgo et al. 2016) (Fig. 1). Binding of GTP or GDP to Tgase2 causes a "closed" conformation unable to catalyze transamidation. In this closed conformation, the C terminus of Tgase2 folds over onto itself and blocks substrate access to the active site of transamidation, located within the catalytic core domain (Gundemir et al. 2012). Increased intracellular Ca^{2+} concentrations reduces the Tgase2-binding affinity for GTP or GDP (Datta et al. 2007), causing the exposure of the active site (Pinkas et al. 2007). This "open" conformation of Tgase2 is enzymatically active of catalyzing protein crosslinking reactions.

In other words, information available at the mid 1990s indicated that (a) Tgase2 had to be considered bona fide a bifunctional enzyme with two distinct activities, the transamidation and the GTP binding and hydrolysis; (b) these activities were mutually exclusive owing to their balanced requirements for activators; and that (c) they served different functions within the cells favoring either survival or cell death. The subsequent development of KO mouse models (De Laurenzi and Melino 2001; Nanda et al. 2001) further supported these conclusions, mostly because they allowed dissection of different functions by investigations in the conditioned animals, despite any lack of major phenotypic disturbance due to suppression of enzyme expression. The conclusion that Tgase2 carries out multiple roles is still valid today and has conducted to the most imaginative definitions of this Tgase as the "nature's glue", the "bête noire", the "Swiss army knife", to mention a few, including "Dr. Jeckill and Mr. Hyde", in a recent review by S. Kojima and associates that was published (Tatsukawa et al. 2016) during the preparation of this Special Issue. Just to say that time was ripe! The same feeling emerged also in the recent GRC on Transglutaminases in Human Disease Processes, held in Girona July 2016.

Research progressed further on the cell biology side taking into account the transmembrane export of Tgase2 to the extracellular space (still a controversial topics) and the relationships between Tgase and the extracellular matrix (with their implications in pathology). Of great relevance were also the observations that the functional roles played by Tgase2 could also be affected by the cellular location of the enzyme (Milakovic et al. 2004) and that a fraction (eventually quite relevant) of the enzyme is located in the

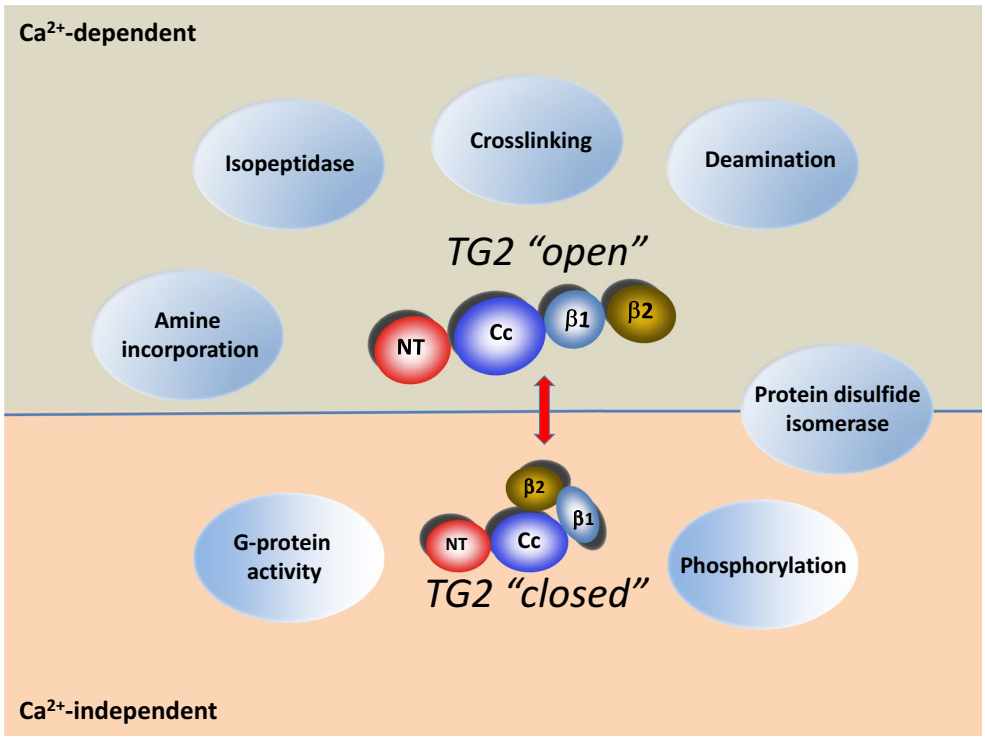

Fig. 1 Structural studies evidenced that Tgase2 may have either a nucleotide-bound closed conformation or a transamidation-competent open conformation. The transamidating and GTP-ase activities of Tgase2 are closely regulated by the availability of calcium and GTP. The different conformational states of Tgase2 may represent the underlying basis for its markedly distinct functions

extracellular space to interact with and to assemble the extracellular matrix (Wang and Griffin 2012), thus contributing to cellular signaling and aggregation, angiogenesis and vascular permeability. This scaffolding activity (as it is usually designated) is likely involved in tissue stability and survival and does marginally require the enzymatic transamidating and GTP metabolic activities already referred to. In this respect, a major throughput achievement has been the demonstration that mutants generated by alternative splicing at the C-terminal region of Tgase2 might display altered regulatory properties (Lai and Greenberg 2013).

But the Tgase enzyme disclosed itself further as a rich fertile mine, although a careful skepticism is always required in dealing with new astonishing "discoveries". This particularly applies to the reports that Tgase2 can display two additional activities, the former as protein kinase (Mishra et al. 2006), the latter as protein disulfide isomerase (Hasegawa et al. 2003). At the eyes of one of us (CMB), these activities still require much research to be definitively proved, although it is interesting to note that these activities do not require calcium and for this reason can potentially play important regulatory functions in physiological cellular conditions. It is now important to define in greater details that these "activities" in the frame of the classic rules of Tgase regulation define specificity of substrates, kinetic parameters, etc., before the bona fide can be accepted as additional intrinsic activities of the protein. For instance, simple transfer of phosphoryl-moieties to protein nucleophiles as alcoholic hydroxyls cannot be accepted as a definitive demonstration of a protein kinase activity since also simple chemical phosphoryl compounds as acetyl-phosphate can phosphorylate proteins (Dallocchio et al. 1982) while simple thiol compounds (e.g., thioredoxin) can display apparent protein disulfide isomerase (PDI) activity based on the classic assay of the rate of renaturation–reactivation of guanidine denatured RNases. A protein with a high number of surface accessible sulfhydryls (this is the case for Tgase that is regulated by disulfide exchange) (Stamnaes et al. 2010) can function in the same way, but for mere chemical grounds not because of an enzymatic activity. Indeed results from experiments by site directed mutagenesis did not yield clear-cut answers. However, the TG2's PDI activity has been proposed to play an important role in mitochondrial homeostasis (Mastroberardino et al. 2006).

The physiology: integrated responses of cells and tissues

In cells, Tgase2 is expressed mainly in the intracellular compartment while as mentioned a fraction of the protein can be released in the extracellular space, under stressful cellular conditions via exosomes (Diaz-Hidalgo et al. 2016), either attached at the cell surface or assembled with proteins of the extracellular matrix (ECM). Although in both locations the transamidating activity is probably normally silent, the enzyme has been claimed to be relevant for various aspects of cellular and tissular responses (Eckert et al. 2014) including opposing effects as proliferation/differentiation *vs* death/autophagy; signal activation and cell responses; interaction with ECM matrix and cell adhesion, migration, cellular anchorage; tissue perfusion and fibrosis (Verderio et al. 2004) to cite a few, like a ying and yang system. These effects attracted much attention during the last years. They have been largely ascribed to different conformations of the Tgase protein, mostly in relation to the contrast between cell death and cell survival (Milakovic et al. 2004; Singh et al. 2016; Tatsukawa et al. 2016). Alternatively, beside arising from direct interventions of the main enzymatic and scaffolding activities of Tgase2, they might depend on the expression of mutant forms of Tgase with altered sensitivity to effectors (Lai and Greenberg 2013) or through the modification of intracellular proteins that trigger differential responses as in the case of the control of NFκB activity by polymerization of IkB (Mann et al. 2006). On the other side, the opposing effects of Tgase on cell functions might relate also to cellular "receptivity", e.g., through the modulation of cellular metabolites like polyamines which are known to influence cellular responses (notably proliferation) (Miller-Fleming et al. 2015), as well as to accumulate inside the cells as protein–polyamine adducts (Folk et al. 1980; Haddox and Russell 1981; Lentini et al. 2004). In addition, in several cell types overexpression of Tgase2 and alternatively its suppression alter features of the cell cycle and these effects are apparently triggered by both native and transamidation inactive mutants as reviewed by Nurminskaya and Belkin (2012) suggesting that the likelihood that these effects are linked directly to protein–protein interaction or alternatively to G-protein activity rather than to the transamidation required to modify the activity of transcription factors like NFκB or Sp1 or the signaling through the TGFβ pathway. In any case, the bases for the pro-proliferative activity of Tgase2 have escaped until now a full explanation, despite the attractive option that this fully depends on nuclear signaling since a minor but still significant fraction of the Tgase protein is located in (or can translocate to) the nucleus (Eckert et al. 2014).

In contrast, more is known on the cell clearing actions promoted by Tgase2. Following the original report by Fesus and associates (1987) that regression of pathologic hypertrophy of liver induced by lead administration depends on increased expression of Tgase2 and apoptosis, several lines of evidence supported the occurrence of programmed cell

death during normal embryonic development (e.g., in kidney and skin) as well in postnatal life, as in the regulation of immune tolerance, thymus regression, glucocorticoid activity, etc. Again, the contrasting roles of Tgase2 in cell survival or death are ascribed to the activation of the GTP signaling or transamidating pathways. Mitochondria are likely involved in these processes, particularly in triggering cell death and Tgase2 has been shown both in a positive and negative fashion in the regulation of this phenomenon (Altuntas et al. 2014). Within this frame, it is hypothesized that Tgase can, by interacting with BAX via its BH3-domain, control the release of cytochrome *c* and apoptosis-inducing factor (AIF) from mitochondria leading to both caspase-dependent and -independent cell death pathways (Rodolfo et al. 2004; Yoo et al. 2012). In this context, the role played by Tgase2 in the process of autophagy which is deficient in cells lacking Tgase2 is also important (D'Eletto et al. 2010, 2012; Altuntas et al. 2015; Kang et al. 2016). Another interesting novel aspect of the presence of Tgase2 at the mitochondrial level is its involvement in the energy metabolism. In fact, it has recently been shown that the enzyme plays an important role in the mitophagy process and in its absence the cells accumulated damaged mitochondria and shift to glycolysis (Rossin et al. 2015; Altuntas et al. 2015; Green et al. 2016).

Conversely, cell damage and death (or alternatively cell growth and tissue repair) can also be induced by stimuli arising from and acting in the extracellular space. The best known example is brought about by damage to cell cultures growing as monolayers. Simple scraping of cell monolayers with a syringe needle or a pipette tip (Upchurch et al. 1991) induces a localized loss of cells in the monolayer and the gap is filled by cells growing from the intact borders of the wound. This requires deposition of and adherence of Tgase2 to ECM proteins, leading to cell motility and proliferation and it is regulated by cellular adherence to, among others, fibronectin, integrins, glycosaminoglycans, syndecans, etc. These interactions apparently do not simply provide a surface for anchorage, but contribute to surface signaling controlling internal messages and thereby cellular responses. Presently, however, the regulation of the transamidating activity of Tgase bound to cell external surface is still unclear and different stimuli affecting the enzyme are continuously identified as in the case of external forces which govern the activity during vascular remodeling in the arterial tree (Huelsz-Prince et al. 2013), as discussed further below. In this optics, interest is raising around the observation that several Tgase substrates can be amidated at glutamine residues by reaction with histamine or serotonin and that these modified proteins carry out distinctive functional properties (Lai et al. 2016). These topics opened also the pathway to consider Tgase2 as a target for therapy and several kinds of inhibitors have been developed, spanning from competitive substrates, up to reversible and irreversible blockers of the active site, to GTP mimics and to stabilizers of conformational states. These aspects have been extensively reviewed (Lai et al. 2008; Badarau et al. 2015; Keillor et al. 2015) along with the effects of the antibodies on the enzyme in a number of autoimmune diseases, notably in celiac disease (Stamnaes and Sollid 2015). Of extreme interest are also the observations about the role of Tgase in antineoplastic therapies because of its intervention in the drug stimulated apoptosis (Budillon et al. 2013). Of great efficacy for instance is the treatment of patients with acute promyelocytic leukemia (but not acute myeloid leukemia) with all-trans-retinoic acid (ATRA) and arsenic tri-oxide, which is fully curative in the vast majority of patients. ATRA can act by both genomic and non-genomic pathways (Schenk et al. 2014) but many of its actions on the differentiating and apoptotic pathways in the granulocyte cell lineage are probably related to regulation of protein expression. ATRA is powerful inducer of Tgase2, so that it might be assumed that the enzyme might be involved also in differentiation and apoptosis of the leukemic cells during the combined therapy, as it was suggested previously in studies on administration of ATRA alone (Benedetti et al. 1996). Notably, several cytokines and probably also vitamin D (Ishii and Ui 1994) can regulate the expression of Tgase2. Unfortunately, the original observation on the effects of vitamin D on Tgase2 expression has not been tested further, despite the growing body of evidence of the pleomorphic physiologic effects of Vitamin D itself (Rosen et al. 2012).

The pathology: protective and aggressive actions of Tgase2

Tgase2 is involved in several disease states (Iismaa et al. 2009), including autoimmune pathologies; CV and neurodegenerative diseases; inflammation and fibrosis; angiogenesis and cancer, frequently with apparently opposing outcomes. We will now examine separately each of these instances, because it must be considered that these contrasting effects might be related by one side to the intrinsic different actions of Tgase2 as a signaling and a protein crosslinking enzyme, by the other to the toxic potential of transglutaminase itself, as well as to the reactive defense response by surrounding healthy tissues. These considerations are central for understanding the opposing effects of Tgase2 in disease pathogenesis as underlined for instance in the case of tumor biology (Kotsakis and Griffin 2007), as authors wonder whether Tgase is "friend or foe".

The story of celiac disease is well known. It is an autoimmune malabsorption syndrome triggered by sensitivity against gliadin with production of antibodies (against

gliadin and deamidated gliadin, as well as Tgase, mostly if crosslinked to other proteins) which stimulate earlier the T cell response and later the B cells with altered secretion of cytokines, production of antibodies, and loss of the normal structure of the intestinal mucosa (Fig. 2). Other pathologies (e.g., anemia, osteoporosis, neurologic complications, epilepsy, recurrent abortion, intestinal lymphoma) also associate with the malabsorption syndrome whose predominant symptom is chronic diarrhea. The specificity of the antibodies for Tgase2 (usually in association with other proteins, chiefly gliadin) as the major autoantigen was recognized in late 1990s by adsorption of patient antisera with intestinal mucosa, immunoprecipitation, and sequencing of the antibody-associated proteins (Dieterich et al. 1997). From the pathologic point of view, the syndrome is characterized by villous atrophy which is probably related to an inflammatory infiltration of the submucosal layers with massive crypt hypertrophy, leading not only to loss of absorption surface but also to mucosal thickening as a result of the hypertrophic response. Antibodies have strong roles not only in diagnosis but also in pathogenesis, although their effects on the enzyme and on mucosal cell responses are still disputed (Lindfors et al. 2009). Many efforts have been dedicated to the identification of the protein regions against which antibodies are raised, with still large debate, another instance of uncertainty—or double face, as you like it—in the transglutaminase field. Great uncertainties are further surrounding the relative involvement of B and T cell lymphocytes with obvious therapeutic implications. Similar Tgase-directed autoimmune reactions have been detected also in other clinical conditions as type 1 diabetes, thyroid diseases, and progressive heart failure, suggesting that transglutaminase-directed autoimmunity might participate in several human diseases, although the case of celiac disease is the best characterized.

The autoimmune inflammatory background stimulated by Tgase2 is observed also in other common pathologies, and can promote fibrotic resolution of long-lasting inflammatory processes in the urinary tract (as in chronic renal diseases), liver (formation of Mallory bodies during cirrhosis), lung (pulmonary idiopathic fibrosis), etc. These degenerative processes usually involve complex circuits linked to chemotactic responses, production and activation of chemokines and Interleukins (which themselves can stimulate further Tgase induction, as for TGFβ) (Kojima et al. 1993), and control of NFκB induction and activation (Mann et al. 2006).

Among the CV pathologies, Tgase has been claimed to be involved special attention due to arterial diseases, since the enzyme is crucial for arterial remodeling following a pressure overload as well as progression of sub-intimal atherosclerotic lesions. In the first instance, the tension that is developed within the muscular layers of the arterial wall is probably the trigger that activates Tgase itself (Huelsz-Prince et al. 2013). Apparently, the contacts between the endothelial surface and the lower fibromuscular layers are

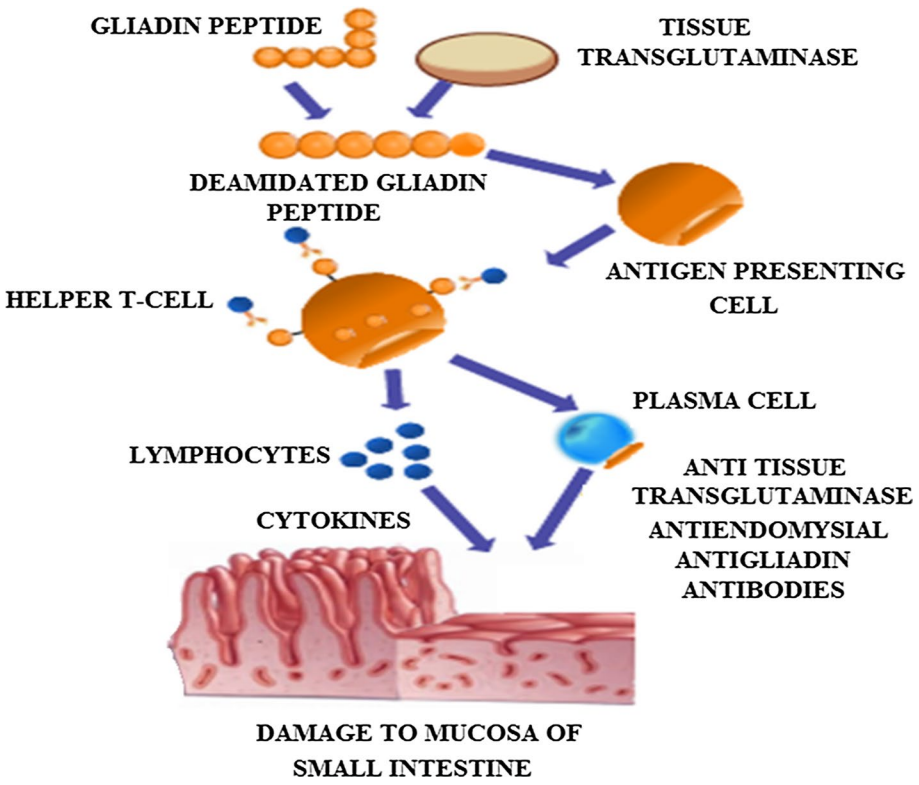

Fig. 2 Celiac disease is an autoimmune malabsorption syndrome, triggered by sensitivity against gliadin, with production of antibodies against gliadin and deamidated gliadin, as well as Tgase, mostly if crosslinked to other proteins, which stimulate earlier the T cell response and later the B cells, with altered secretion of cytokines, production of antibodies and loss of the normal structure of the small intestinal mucosa

crucial in this respect and Tgase is relevant for the regulation of inward remodeling, as well as for the calcification in the layers of the wall (Johnson et al. 2008) and among the endothelial cells, within the atherosclerotic plaque. In this last location, tissue Tgase appears to be a prognostic favorable factor speeding up formation of a fibrotic cap to separate the plaque lipid core from the blood stream in human carotid and coronary artery plaques (Haroon et al. 2001). Conversely, high concentrations of metalloproteinases represent an unfavorable condition predisposing to thrombosis. In different vascular districts, Tgase might not be such a favorable factor as it happens in pulmonary artery hypertension where transglutaminase is induced at high levels of expression by hypoxia and the progressive evolution of the lesions is probably related to signaling by serotonylated proteins (Penumatsa and Fanburg 2014). Again, the positive and negative aspects of transglutaminase activity are mixed. In additon, the microcirculation is sensitive to the effects of Tgase, since it has been reported that inhibition of its activity might suppress angiogenesis, particularly interfering with the function of VEGF that tends to associate with matrix protein, particularly fibronectin (Wang et al. 2013). Observations on the role of the enzyme in microcirculation have obvious relevance in the transcapillary cell migration (Bergamini et al. 2005) as well metastatic tumor spreading and eventually in the balance between tissue regeneration and healing by scarring/fibrosis as reviewed by Nurminskaya and Belkin (2012).

A difficulty in the study and understanding of the functions of Tgase2 is that opposing effects have been reported regarding its roles in the same pathological systems. The role of the enzyme in tumor biology is particularly interesting and controversial, since it has been variously reported that tumors present usually higher concentration/activity of Tgase than the corresponding normal tissues, and that these increases are more prominent in metastatic foci than in the primary lesions. These values correlate chiefly with the differentiation/aggressiveness of the malignancy but high activities are reported also in peritumoral tissues as part of a protective response to limit tumor growth (Haroon et al. 1999). Conversely, the expression/activity of the enzyme has been reported to correlate with sensitivity to chemotherapeutic treatment, to epithelial mesenchymal transition (EMT) and eventually to mediate drug-induced apoptosis. Quite recently, Eckert et al. (2015) obtained evidence that the expression of the enzyme is much higher in cancer stem cells, rather than in the other cancer cell populations, where it apparently promotes survival of the tumor. This behavior is in apparent contrast with the reported roles of the enzyme chemotherapy-induced apoptosis (Budillon et al. 2013) or alternatively in the differentiation-directed therapies such as that happens in melanoma (Lentini et al. 2012). Furthermore, in differentiation-induced BRAF-mutated human melanoma cells, it is not the expression of the enzyme that is increased, but rather its activity, as recently reported (Tabolacci et al. 2016). Another interesting case is represented by clear cell renal carcinoma (CCRC) which is characterized by high expression of Tgase2, whose main target in this tumor is p53, which is crosslinked by the enzyme and addressed for degradation via autophagy (Kang et al. 2016). p53 is a well-known oncogene suppressor, and its degradation prevents apoptosis and favors conversely tumor survival and growth (Kang et al. 2016). In addition, it must be mentioned that also the metabolism of cancer cells is apparently dependent on the action of Tgase2, as it happens in mammary cancer, in which expression and activity of Tgase2 is eventually relevant for triggering the Warburg effect (Kumar et al. 2014; Rossin et al. 2015) through NFκB and HIF signaling and more recently through its interplay with PKM2 a key rate limiting enzyme of glycolysis (Altuntas et al. 2015). The involvement of Tgase2 in EMT and in angiogenesis is of special relevance for the relationships between Tgase2 and tumor growth. Concerning EMT, in the ovarian cancers whose metastatic seeding is promoted by i.p. diffusion of neoplastic cells arising from the surface germinating lining of the ovary, Shao et al. (2009) proved that modulation of Tgase2 by either overexpression or stable knockdown altered the tendency of the cells to undergo EMT and to form tumor spheroids. Obviously, appropriate angiogenesis and vascularization of the tumor tissues are also relevant, particularly for haematogenic dissemination, although it must also be considered that Tgase2 expression is regulated by HIF, since a HIF-RE is present in the Tgase2 promoter. When taken together, these different findings support the idea that the different conformational states of Tgase2 may represent the underlying basis for its markedly distinct functions on pathogenesis.

Another field within massive intervention of Tgase2 for disease progression is represented by chronic neurodegenerative diseases, as well as by trauma. In these pathologies, the enzyme is likely responsible for the deposition of intra/extracellular protein aggregates, the "inclusion bodies" (IB), pathognomonic of Alzheimer (AD), Parkinson (PD), and Huntington diseases (HD) that contribute to cellular toxicity and damage (Lesort et al. 2000). The aggregated proteins of the IB are usually non-native proteins because of oxidative damage (notably in synuclein, in PD) or someway mutated, e.g., with N-terminal glutamine extensions mutations in HD and related poly-Q diseases. Even in this instance, distinct roles of Tgase are apparent: for instance, it might be involved in protein aggregation that converts low Mw highly toxic soluble protein aggregates (Winner et al. 2011) into insoluble aggregates that have limited ability to damage cells (but a definite proof of the involvement of Tgase2 in this process is still lacking), by the other

Tgase2 might exert direct cellular toxicity through G-protein signaling by short forms, following exon-swapping to generate GTP insensitive forms (Citron et al. 2001; Lai and Greenberg 2013). In addition, the Tgase-typic isopeptide bonds have been detected in several neuronal IB, as in amyloid-like aggregates and in AD tangles plaques. The possibility that the activity of Tgase2 is relevant also for the clinical progression of Multiple Sclerosis through modulation of the glial reactivity has been proposed (van Strien et al. 2011). Data from the new PET technology for in vivo detection of Tgase2 presence and activity are waited with interest (van der Wildt et al. 2016).

Conclusions

The literature on Tgases and particularly on type 2 Tgase is growing at a steadily increasing rate, and several papers deal and figure with opposite roles of the protein both on the functional and pathologic point of view for the enzyme in the intra- and in the extracellular location. A recent example is the review that was published by Dr. Kojima and associates (Tatsukawa et al. 2016), while we were setting up this special issue. In this introductory Editorial, we have tried to gather some of the more relevant examples just to present a matter of reflection. We wish everybody for an interesting reading through the research reports that follow.

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

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