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The transglutaminase family: an overview: Minireview article

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Summary. The knowledge that very different processes such as normal and neoplastic cell growth, reproduction and death are dependent on the presence of adequate levels of transglutaminases (TGase: EC 2.3.2.13) and that they are capable of affecting the differentiation and proliferative capability of several cell types, has prompted a multitude of researchers to study these fascinating molecules. In the following overview we intend to summarize the currently known information on the biological significance of these enzymes.

Keywords: Transglutaminases – Cell differentiation – Pathology – Physiology – Cell death

Introduction

The term transglutaminase (TGase) was first introduced by Clarke et al. (1957) to describe the transamidating activity observed in guinea-pig liver. Since this finding, proteins showing TGase activity have now been described following in microrganisms, plants, invertebrates, amphibians, fish and birds. TGases are a widely distributed and peculiar group of enzymes that catalyse the post-translational modification of proteins by the formation of isopeptide bonds. This may occur either through protein cross-linking via ε-(γ-glutamyl)lysine bonds or through incorporation of many primary amines at the level peptide-bound glutamine residues (Folk and Finlayson, 1977). The cross-linked protein products are highly resistant to mechanical challenge and proteolytic degradation, and their accumulation is found in a number of tissues, including skin, hair, blood clotting and wound healing (Lorand and Conrad, 1984). However, deregulation of enzyme activity generally associated with major disruptions in cellular homoeostatic mechanisms has resulted in these enzymes contributing to a number of human diseases, including neurodegeneration, autoimmune diseases, infectious diseases, progressive tissue fibrosis and diseases related to the assembly of the stratum corneum of the epidermis of the skin (Kim et al., 2002; Di Giovanna and Robinson-Bostom, 2003). Moreover the ability of these enzymes to modify proteins and act as biological glues has received much attention by the biotech industries and represents a growing area of TGase research.

In mammals, nine distinct TGase isoenzymes have been identified at the genomic level; however, only six have so far been isolated and characterized at the protein level, after purification either from natural sources or as recombinant proteins. The best characterized enzymes include (a) the circulating zymogen Factor XIII, which is converted, by a thrombin-dependent proteolysis, into the active TGase Factor XIIIa, (plasma TGase) involved in stabilization of fibrin clots and in wound healing; (b) the keratinocyte TGase (type 1 TGase) which exists in membrane-bound and soluble forms, is activated by proteolysis and is involved in the terminal differentiation of keratinocytes; (c) the ubiquitous type 2 tissue TGase (tTG) (Fesus and Piacentini, 2002); (d) the epidermal/hair follicle TGase (type 3 TGase), which also requires proteolysis to become active and, like type 1, is involved in the terminal differentiation of the keratinocyte; the prostatic secretory TGase (type 4 TGase), essential for fertility in rodents; and (f) the recently characterized type 5–7 TGase. All mammalian forms have a good structural homology, are members of the papain-like superfamily of cysteine proteases and are the products of different genes arising from duplication and rearrangement.

All members of this superfamily possess a catalytic triad of Cys-His-Asp or Cys-His-Asn. In addition to the

eight different enzymes described, a further TGase-like protein has been characterized from red blood cells. This protein, named erythrocyte-bound 4.2, has strong sequence identity with the TGase family of proteins, but is inactive because of a substitution of alanine for the active-site cysteine: it forms a major component of the erythrocyte membrane skeleton.

Structural and catalytic features of TGase

The first structural studies on TGases have been performed by high-resolution crystallography on the zymogenic A subunit of plasma Factor XIII, which needs proteolytic processing by thrombin to generate the active dimeric enzyme, revealed that each Factor XIIIA subunit is composed of four domains and that two monomers assemble into the native dimer through in opposite orientation. This organization in four domains is highly conserved during evolution among the TGase isoforms.

Recent structural studies have described the regulatory mechanism of the transamidating activity for the TG2, but can be assumed to act in all the family members. The arrangement of the amino acids of the catalytic centre (Cys277, His 335 and Asp358) in a charge-relay catalytic triad, analogous to that of thiol proteinases such as papain, confers high reactivity on Cys277 to form thioesters with peptidylglutamine moieties in the protein substrate. This high reactivity, in the presence of Ca^{2+}, of Cys277 has been employed to develop a wide range of active-site-directed irreversible inhibitors of the enzyme. In the absence of Ca^{2+}, the TG2 assumes the basic latent conformation and the reactivity of Cys277 is decreased either by hydrogen-bonding with the phenolic hydroxy group of Tyr516 or by formation of a disulphide with a neighbouring cysteine residue, namely Cys336.

The active centre of the enzyme is located deeply within domain 2, hidden from contact with peptidylglutamine substrates by the overlayering of domains 3 and 4, under 'resting' conditions. The Ca^{2+} is the essential activator of the transamidating activity, at the main binding site located at the terminal a-helix (H4) in domain 2. Potential regulatory effects might also occur through the interaction of TG2 with phospholipids and following nitrosylation of cysteine residues by NO donors. It is particularly interesting that lysophosphatidylcholine has the ability to increase the sensitivity of the TG2 to Ca^{2+}, so that the enzyme acquires appreciable activity at near physiological levels of Ca^{2+}, thus broadening the cellular conditions under which the TGase is active. Factor XIII is also inactivated by S-nitrosylation of cysteine residues. Additional informations concerning the regulation of activity in the intracellular compartment confirm the physiological relevance of the ligand-dependent regulatory mechanisms, indicate further possible modulation of intracellular TGase activity through the action of proteinases, since the enzymes are a substrate for both calpain and for caspase 3. TGases are believed to be relatively short-lived proteins, with the half-life of type 2 tTGase calculated to be around 11 h. Very little, however, is known about the regulation of their turnover besides the observation that type 1 and type 2 TGases are subjected to transcriptional regulation by retinoids, steroid hormones and a number of peptide growth factor.

TGases protein substrates

Despite extensive investigations, the question of the identification of substrate proteins of physiological relevance which are post-translationally modified by intracellular TGases is an open one, largely because the products which accumulate *in vivo* or *in situ* in cells and tissues following activation of the enzyme are predominantly highly cross-linked insoluble polymers formed by either direct or polyamine-dependent linkage (Beninati, 1997). Their structure is complicated, so that the identification of the proteins involved in the polymerization process has been very problematic. Probably the best characterized example of a substrate is the way in which the Factor XIIII A cross-links the α-β- and γ-chains of fibrin or its precursor fibrinogen. Despite their great functional relevance, and with the exception of the epidermis, attempts to relate TG2-catalysed protein modification to changes in physiological functions have so far been deceiving and are limited depending on the experimental system. Examples include those involving gain of function such as, stimulation of phospholipase A2 activity, activation of transforming growth factor-β1 (TGFβ1) via cross-linking of the latent TGF β-binding protein-1 (LTBP-1) or loss of function, loss of myosin *in vitro* contractile activity upon addition of TG2-polymerized actin, blockage of protein synthesis upon glutamidation of translation initiation factor 5A ('IF5A') at its unique hypusine residue, inactivation of glyceraldehyde-3-phosphate dehydrogenase and alpha-oxoglutarate dehydrogenase. It is also important to mention that TGase modifies a number of exogenous proteins, including alimentary proteins, like wheat and soya-bean proteins, milk casein and whey proteins (see, for instance, the section on pathogenesis of coeliac disease) and proteins from pathogenic micro-organisms (e.g. Candida albicans surface proteins, envelope proteins and

aspartyl-proteinase from HIV and the hepatitis-C-virus core protein).

For space limitation we do not comment further on this list, although it is evident that a huge number of TGase substrate proteins are involved in cell motility, in the interaction of cells with extracellular matrix structures, and in key pathways of energetic intermediate metabolism (Greenberg et al., 1991; Aeschlimann and Thomazy, 2000; Lorand and Graham, 2003). The importance of defining the TGases substrate and their involvement in cellular function both under physiological and pathological circumstances represent the major challenge for the future for transglutaminase field.

Transglutaminase activity in plant cells

As extensions of the data collected for animal TGases, several directions for future work on plant TGases appear especially promising. The search for new information on plant TGases will continue, with particular emphasis on the control of their expression and biological actions. First, now that the amino acids and nucleotide sequences of almost all the TGases of animal origin have been established, similar efforts with the plant enzymes will help us to understand their function and regulation (Serafini-Fracassini et al., 1995). It is not certain how many distinct TGases are expressed in various plants, but analysis of the genome using molecular cloning techniques may soon clarify their number and structural relations. A second aspect, of TGase structure awaiting clarification and related to the data on the molecular structure of plant TGases, concerns the cellular localization. Finding on the amino acids sequence of the protein will assist in characterizing the anchorage region and the possible mechanism of post-translational modification. Hydrophobic amino acid sequences, fatty acids acylated to amino acid residues and covalently bound phosphatidylinositol-containing phospholipids can contribute to the localization of membrane proteins. In any case, establishing the nature of the anchor will help to evaluate the dynamics of TGase release and its possible intercellular role in carrying out a cross-linking function *in vivo* during cell growth and differentiation.

Physiological functions of TGases

Although the role mof XIII A in blood coagulation as well as the essential role played by TG 1, 3 and probably 5 in the assembly of the cornified envelope of the stratum corneum in terminally differentiated keratinocytes is well established. The search for a physiological function of TGases is certainly not yet over. Most studies dedicated to this issue have tried to extend and attribute general meanings to experiments carried out on relatively narrow and specialized fields. Interestingly, the TG2 knockout mice $(-/-)$ do show symptoms of mild onset of Type 2 diabetes as they age, which is thought to be related to perturbations in insulin release from their pancreatic β-cells as well as development of autoimmunity due to a defects in the clearance of apoptotic cells underlaying the important biological functions played by this enzyme.

Cell death and TGase

The initial report suggesting that TG2 might be involved in cell death by apoptosis came from Fesus et al. (1987), who demonstrated that the levels of enzyme expression and activity correlated with maximum cellular regression found in the livers of rats following induction of hyperplasia. Subsequent studies suggested that TG2 is important in stabilizing the apoptotic cells by intracellular cross-linking, thus preventing loss of intracellular components prior to clearance by phagocytosis. Prior to the acceptance of apoptosis as a distinct form of cell death the involvement of TG2 in a specialized form of cell death had been reported for the human erythrocyte by Lorand and Conrad (1984). Since these initial observations, the involvement of TG2 with apoptosis has been more widely reported. There is also widespread evidence for the up-regulation of the TG2 gene during cell death. However, recent studies have indicated that, by a mechanism thought to involve hyperpolarization of the mitochondrial membrane, TG2 might act as a sensitizer of death stimuli. Probably the most confirmatory evidence indicating that TG2 is important for the apoptotic mechanism comes from the TG2 $(-/-)$ knockout mice, which show the development of an autoimmune phenotype indicating perturbations in apoptotic cell clearance. What is becoming apparent is that expression of TG2 in cells can lead to massive intracellular cross-linking, resulting in cell death if Ca^{2+} homoeostasis is perturbed.

Involvement of TGases in pathology

This topic is attracting much interest and recently has yielded interesting new data with respect to the relevance of TGases in chronic diseases, in particular:

- inflammatory diseases, including wound healing, tissue repair and fibrosis, and autoimmunity;
- chronic degenerative diseases;

– infectious diseases (Hepatitis C, AIDS);
– cancer

Following stress or cellular insult, TG2 is up-regulated and often the enzyme externalized into the matrix. Insult leading to cell damage can also lead to increased TG2 leaking into the matrix.

While this is an example of physiologically oriented involvement of TGases in repair mechanisms, it is also likely that TGases, particularly the TG2, are as a consequence also involved in tissue fibrosis and scarring. Examples include the severe chronic inflammatory states found in liver diseases (cirrhosis and fibrosis, alcoholic hepatopathy and type C hepatitis), and in renal and lung fibrosis. Wound healing requires the involvement of several distinct TGases, which co-operate with each other to finally reconstitute tissue integrità damaged by traumatic or other pathological injuries. Factor XIIIa is clearly involved in the control of blood loss after the traumatic injury of blood vessels, through the stabilization of fibrin during blood clotting, in the activation of platelets, and in the deposition of granulation tissues, which represents the first stable repair to a local lesion. TGases 1 and 3 are particularly involved in repair of the epidermal teguments, in conjunction with TG2, which is probably involved in the angiogenic phase of wound repair as well as in its interaction with and stabilization of the extracellular matrix, possibly through its role as an independent cell-adhesion protein or as an integrin co-receptor.

In the pathogenesis of the chronic inflammatory diseases of the joints, including rheumatoid arthritis and osteoarthritis, has been reported. A major role of the enzyme in many of these conditions is apparently linked to its involvement in the activation of pro-inflammatory cytokines such as TGFb1.

It must also be noted that several autoimmune diseases are characterized by the production of autoantibodies reactive against tTGase. Data in this perspective have been collected mostly for coeliac disease (Sollid, 2002) and Type1 diabetes. Thyroid diseases, and, more recently, systemic lupus erythematosus and Sjšgren syndrome. It has also been suggested that, at least in coeliac disease, the autoantibodies have a pathogenic role, since they interfere with the normal development and differentiation of the intestinal mucosa.

The interest in TG2 immunoreactivity has grown exponentially during the last few years in relation to the pathogenesis and diagnosis of coeliac disease. In the intestinal mucosa of gliadin-sensitive individuals, TG2 is apparently involved in deamidation of glutamine residues in gliadin and in formation of aggregates with gliadin, which are highly immunogenic through local activation of T-lymphocytes. The autoantibodies produced usually belong to the IgA class. Evidence suggests they can exert effects in TG2 function, although evidence for their effects on transamidating activity seems to be controversial.

Probably quite different are the mechanisms whereby TGases are involved in the pathogenesis of several chronic neurodegenerative diseases, which are characterized by the accumulation of highly cross-linked insoluble protein materials. These include senile dementia of the Alzheimer type and the polyglutamine (polyQ) diseases, such as Huntington's disease, rubropallidal atrophy and spinocerebellar palsy. In AD, the expression of TG2 is increased and is also qualitatively altered such that shorter forms of the enzyme are expressed.

In the affected brain, the elevated TG2 activity is manifested by polymerization of a number of proteins, including Ab peptide, beta-amyloid precursor protein and the microtubule-associated tau protein, with formation of neurofibrillary tangles, as well as deposition of amyloid-like materials in the extracellular compartments.

In the polyQ diseases are primarily characterized by transcriptional defects in the substrates with the synthesis of proteins with abnormal poly-glutamine extensions that should represent the sites of TG2-mediated protein cross-linking. This issue is still controversial, since the presence of multiple glutamine repeats directly promotes aggregation in the altered proteins, which tend to polymerize in an enzyme-independent fashion. Recent studies have demonstrated that both the administration of the TG2 inhibitor cystamine to transgenic mice (expressing exon 1 of huntingtin containing an expanded polyglutamine repeat) or their crossino with TG2 KO mice was found to alter the course of the disease in a favourable way, thus providing further evidence for the involvement of TG2 in this disease.

An additional field of active research on the importance of TG2 in human pathology is that of neoplastic diseases. Numerous reports have dealt with these issues, and the general feeling is that tumour cells, when observed *in vitro*, generally have a lower TG2 content than their normal counterparts, contain forms of TG which are identical with those found in normal cells, together with modified forms, which are sometimes inactive, and may differ in their subcellular localization (Beninati, 1995). Tumours usually display a definitively larger proportion of TGase activity in the cell particulate fraction when compared with normal cells, although the absolute amount of enzyme present in this fraction is normally not altered. The decline of TGase activity in tumours is potentially a bad prognostic biomarker and is possibly related to tumour metastatic potential, dictating the ability of the

cells to cross basal membranes and to invade the bloodstream. Given the proposed functions of TGase, reduced enzyme expression and activity in tumours would indeed lead to reduced cell adhesion, increased migration and a less stable extracellular matrix, thus facilitating the initial invasive stage of the tumour. However, reports of increased TGase expression in highly invasive tumours have also been reported, e.g. in the breast, and increased tTGase expression has been found in secondary metastatic tumours. It is also noteworthy that successful induction of TG2 by powerful inducers such as retinoids an effective switch to cell differentiation and apoptotic death. The observation that other synthetic retinoids can be even more active than retinoic acid in inducing tTGase activity and apoptosis in cell lines which are insensitive to the therapeutic effects of retinoic acid has stimulated further research on the application of modified retinoids. It is also now clear that other chemotherapeutic agents of different structure might be as effective as antineoplastic drugs, but their relationships to TG2-related pathways are still controversial. Conversely, host tissues frequently display higher tTGase expression and activity in peritumoral regions, possibly as a local wound-healing mechanism, related to the rearrangement of the extracellular matrix, which may even promote angiogenesis and further spreading of the cancerous cells.

Diagnostic, therapeutic and industrial applications of TGases

This updating on TGase research cannot go unfinished without some mention of the application of TGases as applied biocatalysts in the biomedical and biotechnology fields. This is probably one of the fastest-growing areas in TGase research, as reflected by an increasing number of patent applications filed on TGases. Among the early therapeutic applications of TGases, was the use of Factor XIII substitutive therapy in the rare genetic defects of blood clotting related to loss of the plasma TGase. Most recently the local administration of purified enzymes have been used as an exogenous biological 'glue' to aid in the repair of surgical wounds, fractures and cartilage lesion (Griffin et al., 2002). This practice, employing recombinant rather than extracted enzymes, is still being explored in surgical practice and in the treatment of certain intestinal diseases.

A recent alternative and useful approach is to modulate endogenous TG2 expression, rather than to administer purified enzyme, by means of specific inducers such as the retinoids. This approach is now recognized strategy in dermatological conditions such as acne and, as stated above, in the therapy of selected malignancies *in vivo*. Although studies are still at the experimental stage, additional encouraging results have been obtained in some animal tumours, e.g. melanomas, in which metastatic spread is greatly limited by inducing tTGase activity in either the invading tumour or the host. Earlier studies showing that cell transfection leading to overexpression of TGase in fibrosarcoma cells results in the reduction in tumour growth may have a future application as a tool for gene therapy. The great advantage of such selective therapy, as compared with classic chemotherapy, is its reduced toxicity to normal cells.

Commercial applications of TGase appear continuously at an increasing rate in the TGase research field, for example, in the pathogenesis of infectious diseases and in the development of new strategies in vaccination for bacterial and viral infections. Furthermore, development of bacterial and yeast biofilms frequently involve TGase-like modification of surface proteins. From this perspective we must stress that several bacterial and fungal TGases have been identified, although only one has been extensively purified and characterized. This is the Streptoverticillum morabiense TGase, which does not require Ca^{2+} for activity. This enzyme is commercially available and has found several applications as a biocatalyst in the food, cosmetic and textile industries. Curiously this promising issue has not been the subject of further investigations. Furthermore, it was reported that TG2-mediated polyamidation brought about inhibition of HIV aspartyl-proteinase and that TG2 was crucial in apoptotic clearance of infected T-lymphocytes in the establishment of HIV-associated lymphopenia (Amendola et al., 2002). It has also been reported that TGase-dependent post-translational modification of viral core protein is involved in hepatitis-C-virus cellular replication. This body of information is suggestive of the potential usefulness of pharmacological modulation of TGase activity in these severe viral diseases.

Classic applications of TGase in biotechnology research further include its diagnostic applications for autoimmune diseases, their use in food processing and, more recently, rapid methods of detecting the free ε-(γ-glutamyl)lysine isodipeptide in body fluids, which has the potential to be used as a marker in a number of diseases in which TGases are involved.

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