TGM2 and implications for human disease: role of alternative splicing

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1. ABSTRACT
Alternative splicing is an important mechanism for modulating gene function that accounts for a considerable proportion of proteomic complexity in higher eukaryotes. Alternative splicing is often tightly regulated in a cell-type- or developmental-stage- specific manner and can cause a single gene to have multiple functions. Human Tissue transglutaminase (TGM2) is a multifunctional enzyme with transglutaminase crosslinking (TGase), G protein signaling and kinase activities that are postulated to play a role in many disease states. TGM2 mRNA is regulated by alternative splicing, producing C-terminal truncated forms of TGM2 that are predicted to have distinct biochemical properties and biological functions. In this review, we will discuss how alternatively spliced forms of TGM2 could modulate its roles in cancer, neurodegeneration, inflammation and wound healing.

2. INTRODUCTION
Tissue transglutaminase (TGM2) is perhaps one of the best examples of the functional diversity of a single gene product. Known as a thiol- and Ca2+-dependent transglutaminase enzyme (TGase)(1-3), TGM2 was later found to receive signals from G-protein coupled receptors (GPCRs) including α1-adrenergic (αAR), oxytocin and thromboxane receptors (4, 5). It has also been reported to have a kinase function that can transmit cell membrane signals (6-8). This is an example of how a single molecule can function in a unique manner depending upon its location in the extracellular matrix, cell surface, cytoplasm or nucleus (2, 9). Early biochemical data demonstrated that TGM2 could bind and hydrolyze GTP and ATP (10-13). In addition, TGM2 also functioned as an adhesion molecule involved in an integrin-mediated signaling event (14-16). Recently, TGM2 was reported to display protein disulphide...
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Figure 1. The Crosslinking Reactions Catalyzed by TGM2. A. Inter- or intra-molecular crosslinking between Q- and K-containing peptides; B. Polyamination reaction between Q-peptide and primary amine. C. Deamidation reaction.

Tissue transglutaminase (TGM2) is an enzyme involved in the formation and breakage of disulfide bonds between cysteine residues. Alterations in TGM2 activity and function have been linked to cancer, inflammatory diseases (atherosclerosis), neurodegeneration, tissue fibrosis, autoimmunity, and celiac disease (2, 3, 9). In cancer, overexpression of TGM2 was found to promote cell survival and chemotherapy resistance of the tumor cells (18-20). TGM2 knock out studies in cancer cell lines established that TGM2 expression was associated with cell migration, metastasis, epidermal growth factor (EGF) signaling/cell migration and epithelial to mesenchymal transition (20-22). Since there are four alternatively spliced forms of TGM2, it remains unknown whether full length TGM2 or spliced isoforms can participate in these cellular events. There may be unique structure-function relationships of these isoforms that could play a role in disease processes. In the following section, details regarding function of TGM2 isoforms and their regulation will be discussed.

3. BIOCHEMISTRY OF TGM2

3.1. Transamidation reaction (TGase)

TGM2 belongs to a family of closely related thiol enzymes known as transglutaminases (TGs) that are derived from a common ancestral gene (2, 3, 9). At least eight enzymatically active TGs have been identified (2). TGM2 catalyzes a crosslinking reaction between a specific γ-glutamyl (Q) containing peptide substrate and either a ε-amine group from a peptide-bound Lys (K) residue or a free primary amine (Figure 1). These reactions result in post-translational modifications of proteins that can alter their solubility, structure and function. When a peptide-bound K residue serves as the acyl-acceptor of the reaction, the formation of γ-glutamyl-ε-lysine (isopeptide) bonds results in the formation of either inter- or intra-isopeptide bond (Figure 1a). Many intra- and extracellular proteins have been identified as TGM-2 substrates (2). To date, there are at least 150 substrates reported on TRANSDB database (http://genomics.dote.hu/wiki/index.php/Category:Tissue_transglutaminase). The intracellular substrates that are often cited include K rich nuclear core histones, huntingtin, NFκB, inhibitor alpha and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (23-28). Extracellular crosslinking is important in extracellular matrix (ECM) production and stabilization (3, 29). There are a number of extracellular matrix (ECM) substrates including fibronectin (FN), collagens, osteopontin, nidogen/entactin, vitronectin and osteonectin (3, 29).

When a peptide-bound K residue is not available, the reaction with amines/polyamines results in the incorporation of the primary amino group and the formation of a γ-glutamyl-amine/polyamine bond (Figure 1b; polyamination)(2). Biogenic primary amines, such as putrescine, spermine, spermidine, serotonin and histamine, are potential primary amine substrates of TGase (2, 3). At low pH and when a peptide-bound K-residue and amine are not available, water acts as the acyl-acceptor and the resultant hydrolysis reaction yields a glutamic acid (E) residue (deamidation)(Figure 1c)(2, 30). In celiac disease patients, deamidation is a very specific reaction as glutamine residue in the gluten peptide is deamidated and
patients develop antibody against TGM2 serving as a diagnostic marker for this disease (2). In addition, hsp20 was recently shown to be deamidated at a specific glutamine residue while distinct glutamines were substrates in other types of crosslinking reactions (31).

3.2. Regulation of TGase
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TGase activity is inhibited by GTP, which can be reversed by Ca\(^{2+}\) (10, 32). Under normal conditions, intracellular free Ca\(^{2+}\) (~10\(^{-7}\) M) and GTP (~100-150 \(\mu\)M) are sufficient to keep the TGase in a latent state to prevent cross-linking of proteins with critical function (2, 10). The activation of intracellular TGase activity is a tightly controlled but poorly understood process that can be beneficial or detrimental to cells. Because of high levels of calcium ions (~mM) in the extracellular space, it is generally believed that TGase is constitutively active (2, 3, 9). However, it was recently reported that TGase is not active due to oxidation and the formation of an intra-chain disulfide bond that is activated by thioredoxin (33, 34). Therefore, the activation of extracellular TGase is also controlled by the redox potential of the environment and availability of thioredoxin. S-nitrosylation of TGM2 was found to inhibit its TGase activity (35, 36). TGM2 was found S-nitrosylated in a young aorta but not in an aged aorta indicating there were more TGase activity in aged aorta (35).

Intracellular TGM2 crosslinking events promote inflammation either by activating the NFkB pathway (27, 37) or minimizing inflammation of apoptotic bodies by promoting their clearance (38). In neurodegenerative diseases, crosslinking of proteins can result in the formation of soluble, neurotoxic, oligomeric aggregates and/or insoluble inclusions depending on the type of crosslinking reaction (39-41). The disease-causing proteins that have been shown as substrates include tau protein, α-synuclein, and huntingtin (39-41).

3.3. G protein and kinase functions
In the presence of Mg\(^{2+}\), TGM2 hydrolyzes GTP to GDP, and functions as a G protein (G\(_{o}\)) which can control β-adrenergic-receptor-mediated signaling transduction pathways that lead to PLC\(_{61}\) activation (2, 4, 42). PLC\(_{61}\) activation results in an increase in intracellular calcium which activates the transamidation reaction (2). GTP induces a conformational change that inhibits TGase function by narrowing the active site pocket, this process can be reversed by Ca\(^{2+}\) (10, 32, 43, 44). In contrast to GTP, Mg\(^{2+}\)-ATP binding does not inhibit TGase function but displays kinase activity that phosphorylates histones, retinoblastoma (RB), and P53 (6-8, 10). Kinetic and photoaffinity labeling studies indicated that there are distinct binding sites with the possibility of some common binding residues to both nucleotides (10, 13).

TGM2 binds and phosphorylates insulin growth factor binding protein 3 (IGFBP3) and forms a complex with other proteins with signaling function including ROCK2, glutamate receptor, and GPR44 (6). The peptide fragment associated with IGFBP3 belongs to the N-terminal fragment (AA # 1 to AA 562) of TGM2 (6). Interestingly, TGM2 interacts with protein kinase A and is phosphorylated at Ser\(^{216}\) by protein kinase A (PKA) (45). The phosphorylated-TGM2 creates 14-3-3 binding sites and reduces its TGase activity (45, 46). 14-3-3 protein is an important protein controlling cell cycle progression, metabolism, and apoptosis (47). Silencing TGM2 leads to impaired adhesion and motility of cancer cells by decreasing phosphorylation of AKT kinase (48). It is unknown whether alternatively spliced forms of TGM2 function as G-protein or play a role in cell signaling function.

4. STRUCTURE AND FUNCTION OF TGM2

4.1. TGase active site
X-ray crystallography reveals that TGM2 is composed of an N-terminal β-sandwich (domain I; AA #1-139), a α/β catalytic core (domain II; AA # 140-454), a β-barrel 1 (Domain III; AA # 479-585) and a β-barrel 2 (Domain IV; residue # 586-687)(43, 44) (Figure 2). The TGase active site is composed of a catalytic triad of C\(^{277}\)-H\(^{335}\)-D\(^{158}\) (43), and the rate-limiting step in catalysis involves the formation of a transitional thioester bond between C\(^{277}\) and the Q substrate (Figure 2).

4.2. GTP and ATP binding site
Based on the 3-D structure of GDP-bound TGM2 (pdb: 1kv3), GTP binding involves the amino acid side chains from domains II, III and IV binding to hydrophobic guanine binding pocket (K\(^{173}\), F\(^{174}\), S\(^{482}\), Met\(^{583}\), I\(^{582}\), Y\(^{583}\)), α, β-phosphates (R\(^{478}\), Y\(^{479}\), R\(^{580}\), Y\(^{583}\)), and γ-phosphate (K\(^{173}\) and R\(^{578}\)) (43). 3-D structure of ATP-bound TGM2 also demonstrates that ATP and GDP bind to the same nucleotide binding pocket (49). However, S482 and R580 were found to be involved only in guanine, not adenine binding (49). Mutation at R580 to adenine resulted in almost complete loss of GTP/GDP activity but remained active in TGase function (50) and GTP hydrolysis (unpublished observation). Based on biochemical data, GTP hydrolysis domain is localized to the first 47 AAs of domain II (AA #139-186)(13).

5. BIOLOGY OF TGM2

TGM2 expression is considered ubiquitous, but the distribution and expression levels vary significantly among different cell types. The highest expression level is found in endothelial cells (ECs) and vascular smooth muscle cells (VSMC) (2, 51). TGM2 is localized to the cell-surface, sub-membrane-associated, cytoplasm, and nucleus of the cell (2). In human neuroblastoma cells, 7% of the total TGM2 that is found in the nucleus is associated with chromatin (52). Retinoic acid (RA) induction causes the redistribution of TGM2 in different subcellular compartments (52). The significance of TGM2 induction associated with intracellular translocation to the nucleus and change in intracellular TGase activity is poorly understood (2, 52). Cell surface TGM2 functions as a cell adhesion molecule and interacts with leukocytes (53), a wide variety of ECM adhesion proteins, including integrins, fibrinectin (FN), and GPR56, to increase the
adhesive property of cells (14, 54, 55). Membrane-associated TGM2 is reported to interact with GPR56 and IGFBP-3 (insulin growth factor binding protein) kinase in breast cancer cells and is involved in G-protein and ATP kinase signaling function (6, 55). Cytosolic TGM2 was reported to interact with actin (56), glyceraldehyde 3-phosphate dehydrogenase (GAPDH)(25), importin-α3 (57), EIF-5A (58), histones and huntingtin (23).

There are several mechanisms of how TGM2 is localized to the extracellular space. TGM2 binds to FN (2:1 ratio), a glycoprotein present in plasma and the ECM
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| Table 1. Biochemical properties of different spliced forms of TGM2 |
|-------------------|------------------|------------------|------------------|
|                  | GTPase | GTP Hydrolysis | ATP Hydrolysis |
| tTGH2            | Reduced | 460%*           | ND              |
| tTGH2            | NA     | NA              | NA              |
| tTGv1            | 5%     | 650%*           | 10%*            |
| tTGv2            | 1%     | 650%*           | 10%*            |

ND: Not detected; NA: Not Available. *: Ref. 12; &: Ref. 51

(59), with high affinity (Kd ~8 nM) through its N-terminal \( \beta \)-sandwich domain (Figure 2). FN binding aids in non-classical secretion and localizes TGM2 to the EC surface (54, 61) and the catalytic domain of TGM2 helps stabilize the TGM2-FN interaction (54). Through binding to FN, TGM2 also interacts with \( \beta 1 \) and \( \beta 3 \) integrins and forms stable ternary complexes (14). A recent study indicates that the secretion of TGM2 utilizes the long loop of endosomal recycling pathway and indicates involvement of endosomal trafficking in non-classical protein secretion (62). TGM2 also binds to heparin sulfate proteoglycans (HSPG) with affinity comparable to FN (63). The binding of TGM2 to HSPG also contributes to its externalization (63, 64). In addition, cancer cells can also utilize microvesicles containing TGM2 and FN to transfer proteins into recipient cells (65).

Increased TGase activity is a common feature of several inflammatory diseases. The TGM2 promoter contains a NF\( \kappa \)B binding motif (66) and is induced by NF\( \kappa \)B. Several inflammatory cytokines including IFN-\( \gamma \) (67) and TGF-\( \beta 1 \) (68) are known to up-regulate TGM2. TGM2 is also involved in the conversion of latent to active TGF-\( \beta 1 \) whereas the TGM2 itself is induced by TGF-\( \beta 1 \), thus forming a positive feedback loop between the two proteins. TGFB1 expression down regulates inflammatory and autoimmune responses (69).

TGM2 is also induced by RA and inflammatory cytokines (IL-6, TNF\( \alpha \)), epidermal growth factor (EGF), and by various stimuli such as UV light, oxidative stress, and viral infection (2, 70). By mechanisms that are currently unknown, TGM2 mediates the expression of the gp91\( ^{phox} \) subunit of NADPH oxidase expression in PMNs, a gene product that is essential for oxidative killing by generation of superoxide anions (71). These data demonstrate that the induction of the TGM2 is associated with a host response to various stimuli that lead to cell differentiation, inflammation and immune defense.

In earlier studies, we observed co-expression of the TGM2 and active TGF-\( \beta 1 \) at sites within wounded regions and at sites of inflammation (72-74). Our group has shown that direct application of recombinant TGM2 to a mammary adenocarcinoma using a dorsal skin flap window chamber model resulted in increased levels of collagen around the tumor, resulting in fibrosis (72). There data demonstrate the role of TGM2 in the wound healing response and abnormal TGM2’s activity could lead to tissue fibrosis.

6. ALTERNATIVE SPliced FORMS OF TGM2.

6.1. Importance of C-terminal residues of TGM2

TGM2 mRNA has been reported to be regulated by alternative splicing, producing shorter forms of TGM2 that are predicted to have distinct properties (11, 75-77). There are a total of 4 alternatively spliced forms of human TGM2 in the literature. These include tTGh (also called TGase-S), tTGH2 and tTGv1 and tTGv2 (51, 75, 76) (Figure 3). The roles of different spliced forms of human TGM2 in vivo remain unclear. The difference between TGM2 and its spliced isoforms is their C-terminus. All spliced forms lose their C-terminus to different extents (see the following section for more details). The C-terminus of TGM2 was found to bind to GPCRs including the \( \alpha 2 \)-adrenergic receptor and an N-terminal adhesion domain of GPR56, a receptor involved in migration and adhesion of tumor cells (78). The C-terminus (AA # 657-664) also contains nuclear export signal peptide sequences that is important for intracellular localization (see below) (79). C-terminus is important for protein stability and GTP/ATP binding (51). Part of the Heparin binding sites are localized at the C-terminal amino acid (AA) # 598-602 (80). In addition, the C-terminus (AA # 661-672) of TGM2 is involved in PLC\( \delta 1 \) binding (5). PLC\( \delta 1 \) is an isoform of PLC that generates inositol 1,4,5-triphosphate (IP3) and diacyl glycerol, which mobilizes intracellular Ca\( ^{2+} \) from the endoplasmic reticulum (ER) (5). The activity of PLC\( \delta 1 \) is suppressed by the interaction with TGM2 (42). When GTP binds to TGM2, PLC\( \delta 1 \) is released from inhibition (42).

6.2. tTGH and tTGH2

In human erythroleukemia cells, 63 kDa (designated tTGh) and 37-kDa (designated tTGH2) N-terminal fragments of TGM2 were reported (77). tTGh and tTGH2 mRNAs are generated by common alternative splicing events through incorporation of intron X and V1 (Intron retention mechanism) (81) and encode proteins with 548 and 349 amino acid residues, respectively (76, 77) (Figure 3). Both mRNAs have alternative polyadenylation site utilization (Figure 3). tTGh, which lacks the C-terminal 138 amino acid residues of TGM2, sometimes referred to as short isoform (S-form, or TGase S) in the literature (82, 83) has been characterized and was found to be up-regulated in brains of Alzheimer’s patients. tTGh remains uncharacterized. These two forms were not found in human vascular endothelial and smooth muscle cells and may reflect changes derived from leukemogenesis (51).

tTGH is estimated to display ≤ 5% residual TGase activity based on data from the recombinant C-terminal truncation mutant (M' \( -S^{538} \) ) expressed in E. coli (12) (Table 1). The rate of GTP hydrolysis activity of tTGh was found to increase (11, 12), however, it was found to have weak affinity to GTP since the binding was not detectable using photoaffinity labeling (84). A similar observation with high GTP hydrolysis rate (6-7 fold increase) but with low GTP binding was also reported for tTGV1 and tTGV2 (51) (Table 1). It should be noted that low measurable GTP binding does not mean reduced GTP hydrolysis (GTPase) activity. Since TGM2 has high affinity for GTP/GDP as demonstrated by co-purification of GDP and TGM2 in crystal (43), TGM2 is likely to have
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slow exchange rate for GTP and GDP; i.e., TGM2 has a prolonged “docking time” for the replacement of GDP with a new GTP molecule. Therefore, with weaker GTP binding for tTGH, tTG\_V1 and tTG\_V2, they have much higher rates of GTP hydrolysis (51). The significance of high hydrolysis rate of these TGM2 isoforms remains unknown.

6.3. tTG\_V1 and tTG\_V2

Two cDNAs encoding additional C-terminal truncated forms, tTG\_V1 and tTG\_V2 were identified from human aortic vascular smooth muscle cells (51). tTG\_V1 and tTG\_V2 are composed of 674 and 645 amino acid residues that share identical N-terminal 622 amino acids with TGM2 with alternate 52 and 23 amino acids at the C-terminus that translates into proteins with the predicted M\_w of 75 and 70 KDa, respectively (Fig 3). tTG\_V1,2 mRNAs were synthesized by a rare splicing event utilizing alternate splice sites within exons XII and XIII of the TGM2 gene, respectively (Fig 3). Alternative polyadenylation sites are

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**Figure 3.** Schematic diagram of TGM2 and different isoforms and the mechanism of splicing. A. The grey box represents TGM2 protein sequences, open box and other shaded boxes represent alternate amino acid (AA) sequences due to change in reading frames. The number represents the amino acid residue. B. The mechanism of splicing. The exon structure of TGM2 gene is depicted. Proposed processing events are numbered 1 to 5. The products of these processing events are illustrated. Events 1-2 are splicing events; events 3-5 are polyadenylation events. The length of message RNAs are not drawn to scale.
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also utilized (Figure 3). The loss of C-terminal 52 AA residues in tTG1,2 reduces GTP binding, enhances GTP hydrolysis, renders the variants insensitive to GTP inhibition, and results in <10% residual TGase activity (51). The data suggests that C-terminal residues are important in GTP binding and GTP hydrolysis. The reduced affinity for GTP could allow tTG1,2 to escape regulation by GTP and exhibit TGase activity when there is a transient increase in Ca²⁺ levels. In addition, the reduced affinity for GTP may allow these isoforms to have higher GTP hydrolysis rate and this could have implications that remain to be determined.

HEK293 cells transfected with vectors expressing tTG1 and tTG2 demonstrated a 28- and 5-fold, respectively, reduction in the levels of protein expression demonstrating that the deleted C-terminal 52 AA is important in stabilizing and promoting the half-life of TGM2 (51). This is consistent with the observation that S171E/TGM2, a point mutation GTP binding-deficient mutant, was poorly expressed in NIH3T3 cells (85, 86). Another GTP-deficient mutant, rat R579A/TGM2 (correspond to R580 in human TGM2), also had relatively lower expression levels than wild-type TGM2 in SH-SY5Y cells (87). Its GTP-bound conformation may exclude TGM2 from the intracellular degradation pathway. This also warrants further investigation.

There was unique expression and localization of tTG1,2 compared with TGM2 in human umbilical vein endothelial cells (HUVECs), vascular smooth muscle cells (VSMC), and leukocytes. In HUVECs, TGM2 mRNA was expressed at 32- and 194-fold higher levels than tTG1 and tTG2, respectively, while in HL-60 and leukocytes (MN and PMN cells), the expression of TGM2 is decreased and the difference between TGM2 mRNA and tTG1 (tTG2) mRNA are only 2.5-fold difference (51). At the protein level, tTG1 is either expressed at much higher or similar levels in human leukocytes and HL-60 cells, respectively (51). This could be due to the stability of mRNA or because TGM2 protein is decreased in HL-60 and leukocytes. The abundance of tTG1,2 and distinct intracellular expression patterns in human vascular cells and leukocytes indicates these isoforms may have unique physiological functions.

In rats, the GTP-independent isoform (s-TGN, also lacking the C-terminal 52 AA) was isolated upon stimulation with inflammatory cytokines (IL - 1β and TNFα ) and was induced to high levels in inflammation and in rat brain injury after cerebral and spinal cord injury (88-92). Therefore, the induction and expression of tTG1,2 and S-TGN could be considered as a response to tissue injury. Similar to tTG1 and tTG2, the spliced site of rat S-TGN is also localized at amino acid #622 and generates an alternate 30 amino acids at the C-terminal end. This isoform was shown to be less sensitive to GTP-mediated inhibition of TGase activity (88, 89); however, the TGase activity of rat s-TGN with respect to full-length TGM2 was not reported (88, 89).

7. IMPACT OF ALTERNATIVE SPLICING OF TGM2 IN VARIOUS DISEASES AND BIOLOGY

TGM2 is involved in many diseases and biological processes (as listed and discussed below). The contribution of these splicing isoforms of TGM2 in these diseases and processes warrants further investigation.

Overall, the loss of C-terminal residues in the splice variants may affect their ability to form complexes with other proteins. Recently, TGM2 secreted in the ECM was found to serve as a ligand for GPR56, an orphan G-protein coupled receptor that is involved in suppressing tumor growth and metastasis (55). The N-terminal domain of GPR56 was found to bind to the C-terminal β-barrels of TGM2 (55). Since the C-terminal β-barrels are required for TGM2 to bind GPR56 (55), tGHI, tGHL2, tGVI, and tTG2 may no longer serve as adhesive ligands for GPR56.

The C-terminal AAs (#661-672) of TGM2 are involved in PLCδ1 binding (5). The activity of PLCδ1 is suppressed by the interaction with TGM2 (42). When GTP binds to TGM2, PLCδ1 is released from inhibition (42). PLCδ1 shuttles between the cytoplasm and the nucleus (93, 94). By lacking critical C-terminal AAs, tTG1 and tTG2 may have a defect in PLCδ1 binding and be unable to adopt a GTP-bound conformation, suggesting that PLCδ1 function, including its nuclear activity might be enhanced. The interaction of TGM2 with PLCδ1 and/or importin-α3 may be an important mechanism that regulates its distribution between the cytosol and nucleus (2, 5, 57). Thus, the potential loss of interaction with PLCδ1 may contribute to the unique pattern of intracellular localization of the splice variants.

A putative nuclear localization export signal (NES) was localized at the C-terminus of TGM2 (AA # 657 to 654) (79). Nuclear TGM2 was previously shown to be induced in the nucleus of ethanol or free fatty acids treated hepatic cells. Nuclear TGM2 was shown to crosslink and inactivate the Sp1 transcription factor, leading to caspase-independent hepatic cell death in a tissue culture system. Recently, they showed that ethanol or free fatty acids also induced the accumulation of tGHI in the nucleus, as it lacks the NES. The physiological significance of accumulation of tGHI in the nucleus remains to be determined (79).

The loss of the C-terminus also affects the ability of isoforms to induce cell differentiation (98). TGM2 was unable to induce neuroblastoma cell differentiation, however, over-expression of tGHI (TG2-5 in their study) was shown to induce neuroblastoma cell differentiation (98). The ability of tGHI to induce neuroblastoma cell differentiation was due to its deficiency in binding to GTP as another GTP binding deficient mutant R580A also showed the same activity.

Recently, the expression of TGM2 and various spliced isoforms has been analyzed using real-time PCR in various normal and cancer cell lines (99). They found in normal cells that the expression of spliced forms are lower and correlated well with the expression of TGM2.
However, there is more variability in the relative expression of different isoforms which did not correlate well with the expression of TGM2. Specifically, there was preferential expression of specific TGM2 isoforms (tTGH and tTG2) from melanoma cell line and prostate cancer biopsies (99). This data is consistent with other reports that alternative splicing is a more active process in cancer cells (100).

7.1. Epithelial-Mesenchymal transition (EMT)

EMT is a critical process in cancer progression (101). EMT is characterized by breakdown of cell junctions and loss of cell polarity, rendering epithelial cells motile and invasive (101). TGM2 positively influences the development of EMT through at least two mechanisms. First, it cross-links the large latent form of TGFβ to the extracellular matrix (102), this may concentrate or release bioactive TGFβ, an inducer of EMT. TGM2 and TGFβ reciprocally induce each other as part of an auto-stimulatory loop, thus emphasizing the role of TGM2 in the EMT process. Second, TGM2 activates NFκB, a recognized EMT inducer, by cross-linking and polymerizing the inhibitor of NFκB, IκBα, leading to its proteasomal degradation (103). Inhibition of TGM2 expression by siRNA blocks EMT induction (19). In addition to affects on TGFβ and NFκB (19, 104), TGM2 may affect tumorigenic properties by enhancing integrin binding to ECM components and activating focal adhesion kinase and Src (21). Using breast cancer cell lines as model systems, Kumar et al. suggested TGase activity is not essential for EMT, while GTP binding was important since an overexpression of a GTP binding deficient mutant TGM2/R580A failed to induce EMT phenotype (20). Since R580A/TGM2 is deficient in GTP binding but has enhanced GTP hydrolysis activity (unpublished observation) suggesting it has increased G-protein signaling function, a mutant with no GTP hydrolysis activity will be needed to investigate its role during EMT. All spliced forms (except TGH2) of TGM2 are deficient in GTP binding and have residual TGase activity, however overexpression of these spliced forms are not postulated to induce EMT.

7.2. Autoimmunity

Intracellularly, TGase activity is important in apoptotic body formation and preventing the release of intracellular proinflammatory substances. Apoptotic bodies also have an anti-inflammatory effect by inducing the anti-inflammation cytokine TGF-β (105). Defects in TGM2 lead to abnormalities in clearing apoptotic cells and cause immune dysfunction and inflammation (106). TGM2- mice fail to activate TGFβ , have delayed clearance of apoptotic cells and have evidence of autoimmunity (38). All spliced forms have residual TGase activity and overexpression of these spliced forms is postulated to have increased chances of getting autoimmunity.

7.3. Neurodegenerative diseases

TGase-mediated cross-linking is postulated to contribute to several pathologic hallmarks in neurodegenerative diseases including neuroinflammation, accumulation of insoluble protein inclusions, and proteasome dysfunction (107). TGM2 is implicated in Huntington’s disease (HD), Parkinson’s and Alzheimer’s disease. Although intracellular TGase activity is tightly regulated, it is possible that, TGase becomes activated by repeated responses from intracellular injuries, including oxidative stress mediated by mis-folded proteins such as expanded polyQ proteins which results in small transient rises of Ca2+ concentration (108). This allows for the gradual accumulation of TGase-mediated cross-linked products over a long time period which would provide further neurotoxic crosslinked products. This finding may further explain why these diseases manifest so late in life.

Since tTGH, tTG2 and tTG2 are not readily inhibited by GTP, they could be readily activated by any transient intracellular Ca2+ spike stimulated by oxidative stress (95) or in diseases associated with mitochondria dysfunction (96, 97). Although tTG2 has less TGase activity, the constitutive crosslinking activity could allow for the gradual accumulation of cross-linked products over a long time period, which could result in further cellular injury. The TGase activity was reported to function as a cofactor to crosslink IκBα and activate NFκB in the nuclei of brain astrocytes and BV-2 microglia (26, 27, 109). As tTGH, tTG2 and tTG2 have residual TGase activity, they are postulated to have reduced ability in activating the NFκB inflammatory response and this requires further investigation.

7.3.1. Huntington’s disease

In neurodegenerative diseases, TGM2 is known to catalyze the inter- or intra-molecular crosslinking of tau protein, α-synuclein (SYN), and huntingtin forming soluble oligomers, while unmodified or polyaminated disease proteins produced insoluble inclusions (39-41). In vivo data have validated TGM2 as a target for inhibition in HD. Data from cross-breeding TGM2 knock-out (TGM2 KO) (TGM2 -/ ) and two different models of HD (R6/1 and R6/2) mice (see below)(110, 111) and pharmacological (cystamine) inhibition (112, 113) all show beneficial effect of inhibiting TGM2’s function. Because soluble crosslinked complexes are transient species and not readily detected in vivo, they have been ignored in the past. However, there is recognition that soluble and diffusible high molecular weight oligomeric complexes (or micro-aggregates) are the neurotoxic intermediates in neurodegenerative disorders (114, 115), while the insoluble inclusions could represent a non-toxic pool of insoluble proteins. Moreover, isopeptide bonds are protease-resistant, cannot be easily degraded by the intracellular degradation pathway, may cause proteasome malfunction and trigger neuronal cell death (116).

7.3.2. Parkinson’s disease (PD)

The role of TGM2 in Parkinson’s disease (PD) was based on several findings including: 1) the discovery of synuclein (SYN) protein as an in vitro and in vivo TGM2 substrate (117, 118); and 2) the increased levels of TGM2-catalyzed cross-links co-localized with SYN in Lewy bodies, which correlated with the development of PD in patients (118). TGM2 interacts directly with SYN both in vitro and in cell models (117, 119). Andringa et al.
demonstrated that SYN is indeed a substrate for TGM2 in vivo (118). Increased levels of TGM2-induced intra- and intermolecular cross-linked SYN are observed in PD brains, which suggest that this cross-link precedes further aggregation of SYN into Lewy bodies (118). In addition, crosslinked products of ubiquitin, hsp27, and SYN were also reported in Alzheimer’s brains (120). As the crosslinked products contain protease resistant isopeptide bonds, they were postulated to interfere with ubiquitin-proteasome degradation pathway of unfolded proteins (120). These studies suggest that inhibiting the TGM2’s cross-linking might prevent the development of Lewy bodies, and thus the development of PD.

7.3.3. Alzheimer’s disease (AD)
AD is characterized by the formation of extracellular neurotoxic aggregates consisting of amyloid-beta protein, or intracellular neurotoxic aggregates consisting of hyperphosphorylated tau (121). Both amyloid-beta and tau have been shown as good in vitro substrates for TGM2 (121). Phosphorylated tau accumulated in neurofibillary tangles, as well as non-phosphorylated tau are substrates for TGM2 (121). In addition, TGase’s crosslinking of angiotensin II AT_2 receptor causing dysfunctional G-protein signaling was shown to enhance the development of neurodegenerative symptoms in transgenic animal model of Alzheimer’s disease (122).

tTGH was shown to be up-regulated in Alzheimer’s disease (AD) brains. However, it is not established which brain cells produced this protein and to what extent this form was derived from leukocyte infiltration can not be determined. Quite interestingly, tTGH was shown to be predominantly induced in TNFα and Doxorubicin-induced in NIH3T3 cells (84). This could be due to cellular factor(s) that favor the production of tTGH mRNA and/or the stability of tTGH. In contrast to the role of TGM2 in protecting cell death, increased apoptotic cells were found in NIH3T3 and SKBR3 breast cancer cells transfected with plasmid DNAs encoding tTGH (84). The pro-apoptotic effects were not dependent on its TGase activity because mutants with active site Cys277 mutated did not produce the same effects, nor was it due to GTP binding because tTGH has little or no GTP binding ability (84). It was the tendency of tTGH itself to form aggregated oligomer that triggers the cell death (84). Based on these data, one would expect over-expression of tTGH to increase cell death of brain cells and the possibility of developing Alzheimer’s disease. However, this hypothesis remains to be tested.

7.4. Inflammatory diseases
Increased TGase activity is a common feature of several inflammatory diseases. TGM2 is involved in enhanced inflammation by participating in an inflammatory loop with the “master switch” for inflammation, NFκB, and functions by reducing free I-KappaBα, leading to the translocation of free NFκB into the nucleus (26, 27, 103). In addition to NFκB, several inflammatory cytokines including TNFα, IFN-γ (67) and TGF-β-1 (68) are known to up-regulate TGM2. In addition, through intramolecular crosslinking and polyamine incorporation, TGM2 can modify PLCA2 (sPLA2), an enzyme that releases arachidonic acid from cell membrane glycerophospholipids, leading to the synthesis of inflammatory eicosanoids. Both modifications significantly augmented the activity of sPLA2 (123-125). Since all spliced forms have reduced TGase activity, abnormal expression of these isoforms will have decrease ability in activating NFκB mediated inflammatory pathway.

7.5. Wound healing and fibrosis
TGM2 can be viewed as a micromolecular suturing enzyme (biological glue) that enables tissues to resist proteolytic digestion and acquire enhanced mechanical strength (126). A stable ECM constitutes an important element to promote normal angiogenesis and wound healing (30). TGM2 is secreted by cells and is bound to fibronectin (FN) in the ECM (127). In addition to FN, many ECM proteins including collagen, fibrinogen, fibrin, laminin/nidogen, osteopontin and vitronectin are also substrates of TGM2 (128).

TGM2 expression and activity were increased very early during wound healing, which demonstrate that the TGM2 was activated in cells that were migrating into the fibrin clot and/or remodeling the ECM (72). TGM2 expression occurred in association with TGF-β, TNF-α, IL-6, and VEGF production in the wound (72). How and when TGM2/TGase becomes activated outside the cells and what role it plays in cell biology remains poorly understood. TGM2 can also influence ECM biology by localizing cytokines and protease inhibitors (ECM stabilization phase) to the matrix (129-131). TGM2 can crosslink elastin (a potent inhibitor for elastase) and alpha_2-antiplasmin (a potent plasmin inhibitor) to ECM molecules (130, 132, 133). TGM2 binds to beta-1 and beta-3 integrins (53, 61, 134-136) and functions as a co-receptor to promote cell adhesion (136). Moreover, TGM2 is involved in the conversion of latent to active TGFβ (137, 138). Active TGFβ can also induce TGM2 gene expression leading to further TGF activation which ultimately leads to accumulation of a protease resistant ECM (139, 140).

Under pathological conditions, TGM2 exerts its effects at different phases of wound healing, leading to fibrosis. In the initial phase (trigger/inflammation), TGM2 gene expression is induced by inflammatory cytokines (IL-1, IL-6, TNF-alpha and TGFβ) (137, 141-144) as a response by damaged tissues to attract inflammatory cells (137). TGM2 can also serve as a receptor to recruit T cells into tissues which would further amplify injury responses (145). It is also possible that tissue injuries produce excessive TGFβ resulting in TGM2 up-regulation and extensive ECM crosslinking which leads to a microenvironment in tissues that promotes fibrosis.

These spliced isoforms will have reduced ability in crosslinking ECM substrates as they have reduced TGase activity, which may have effects in slowing down the wound healing process. However, this hypothesis remains to be tested.
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7.6. Cell adhesion

TGM2 also functions as an adhesion molecule that contributes to cell-cell and cell-ECM interactions (2). The folding of the N-terminal β-sandwich and two C-terminal β-barrel domains of TGM2 is similar to the immunoglobulin like (IgF)-folding domain, a major family of adhesive proteins that are predicted to be involved in protein-protein interaction (146, 147). Several adhesive proteins including FN, ICAM, VCAM and cadherin (148), all have a similar IgF fold (149). TGM2 binds to FN (2:1 ratio), a glycoprotein present in plasma and the ECM (59), with high affinity (Kd ~8 nM) (60) through its N-terminal β-sandwich domain that aids in the non-classical secretion and localizes TGM2 on the EC surface (14, 54, 150). The catalytic domain of TGM2 helps stabilize the TGM2-FN interaction (54). Through binding to FN, TGM2 also interacts with β1 and β3 integrins which forms stable ternary complexes (14). In fibroblast and monocytic cells, TGM2 also interacts directly with β1 and β3 integrins and associates with the cell surface (14, 15). All spliced forms retain N-terminal β-sandwich domain and are expected to contribute to adhesive function, as TGM2. The role of different spliced isoforms of TGM2 remains to be investigated in this process.

In summary, alternative splicing is an important mechanism for modulating gene function that accounts for a considerable proportion of proteomic complexity in higher eukaryotes. Alternative splicing is often tightly regulated in a cell-type- or developmental-stage- specific manner and can change how a single gene has multiple functions. These C-terminal truncated forms of TGM2 could play an important role in the pathogenesis of various diseases. All these isoforms lack the NES signal sequences, thus they could be localized in nucleus (as demonstrated for tTG1 and tTG2) to perform abnormal function. As they are no longer regulated by intracellular GTP, they could be easily activated by the repeated rise in Ca2+ seen in neurodegenerative (or other) diseases; which could contribute to the cross-linking of disease-causing proteins.

8. ACKNOWLEDGEMENT

This research was funded in part by NIH grants HL072184 (CSG) and NS050541 (TSL). The critical reading of the manuscript by Joseph Meserve is highly appreciated.

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**Key Words:** Alternative Splicing, Transglutaminase, Cancer, Neurodegenerative, Wound Healing, Review

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