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Role of protein modifications mediated by transglutaminase 2 in human viral diseases

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1. ABSTRACT

Transglutaminase 2 (TG2) belongs to a family of calcium-dependant enzymes that catalyze transamidation reaction, producing polymerized, polyaminated or deamidated proteins. Recently, a growing number of viral proteins as well as cellular proteins with which they interact have been found to be modified by TG2, suggesting a novel function for TG2 in viral pathogenesis. This review summarizes the results of relevant research, examines the mechanisms underlying TG2 function in host-virus interactions and proposes a model for viral pathogenesis involving TG2.

2. INTRODUCTION

Transglutaminases (EC 2.3.2.13) are a family of calcium-dependent enzymes that catalyze post-translational modifications of proteins involving cross-linking of proteins, incorporation of polyamines into proteins and/or deamidation of proteins (1). Among the eight transglutaminase isoenzymes identified in the human, TG2 is particularly interesting because it is ubiquitously expressed and localized both in subcellular and extracellular sites (2). In addition, TG2 is a multifunctional enzyme that has GTP hydrolyzing activity (2). However, TG2 knockout mice are normal with no physiological or

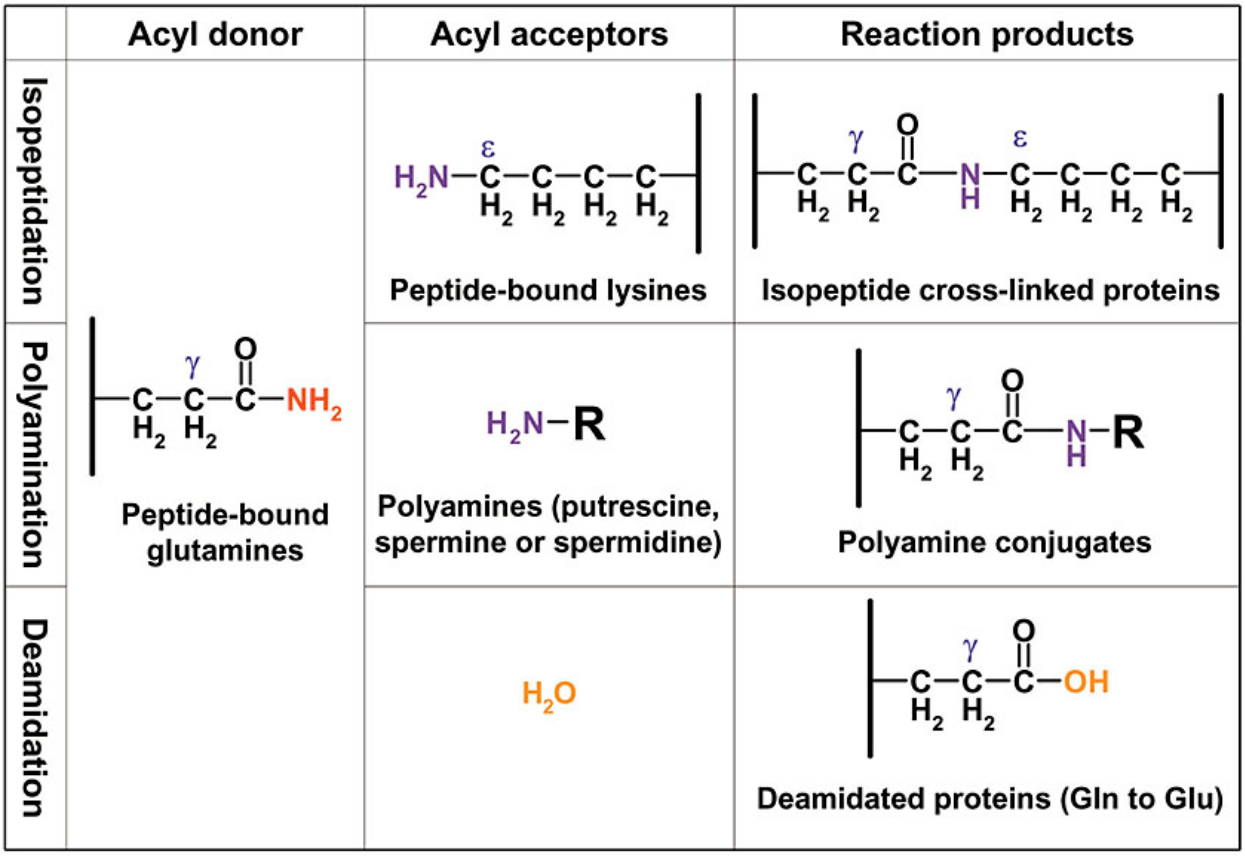

Figure 1. Post-translational modification catalyzed by TG2. Protein modifications are classified as isopeptidation, polyamination, and deamidation depending on acyl acceptor substrates. Isopetidation occurs in either inter-molecular or intra-molecular fashion. Polyamination also produces inter- or intra-molecular cross-linked proteins by acting as a bridge between gamma-carboxyamide of glutamine residues.

developmental defects (3-4). What then are the physiological implications of TG2-mediated protein modification? Studies involving substrate analysis have provided some understanding of physiological roles of TG2. A wide range of proteins have been identified as TG2 targets and substrates for post-translational modification (5). Several viral proteins as well as cellular proteins with which they interact in virus-infected cells have been recently shown to be substrates for TG2, suggesting a possible role for protein modifications caused by this transglutaminase in viral pathogenesis. Analysis of altered function of these proteins caused by TG2 has indeed provided new insights into host-virus interactions that underlie viral infection as discussed in this review.

3. ENZYME CHARACTERISTICS OF TG2

A discussion of the salient enzymatic features of TG2 is necessary in order to understand the biological significance of protein modifications resulting from transglutaminase action during viral infections.

3.1. Nature of modification depends on acyl acceptor substrates

The acyl transfer reaction catalyzed by TG2 occurs on the catalytic triad composed of Cys-277, His-335 and Asp-358 (1-2, 6-7). Nucleophilic attack of the SH group of Cys-277 on the gamma-carboxyamide of the glutamine residue (acyl donor) produces a thioester acylenzyme intermediate. Accompanied by the release of ammonia, subsequent entry of an amine nucleophile (acyl acceptor), the epsilon-amine group of a peptide-bound lysine or the primary amine group of a polyamine, or water attacks the acylenzyme, generating cross-linked, polyaminated, or deamidated proteins (Figure 1). Thus, acyl acceptor substrates determine the type of protein modification. However, a structural motif or a mechanism that selects the reactive glutamine residues or acyl acceptors is not known. Accordingly, it is clear from substrate analysis that type of modification of proteins should be confirmed in intact cell as well as *in vitro*.

3.2. Intracellular TG2 activity does not correlate with the protein level

Quantitating N [epsilon]-(L-gamma-glutamyl)-L-lysine or N [epsilon]-(L-gamma-glutamyl)polyamine isopeptide is the only way to prove the physiological activity of TG2 (8). However, this approach does not permit the assessment of the *in situ* activity of TG2 due to practical difficulties and the technical limitations. Recently, it was shown that *in situ* activity of TG2 could be monitored conveniently using activity-based probes (9). In

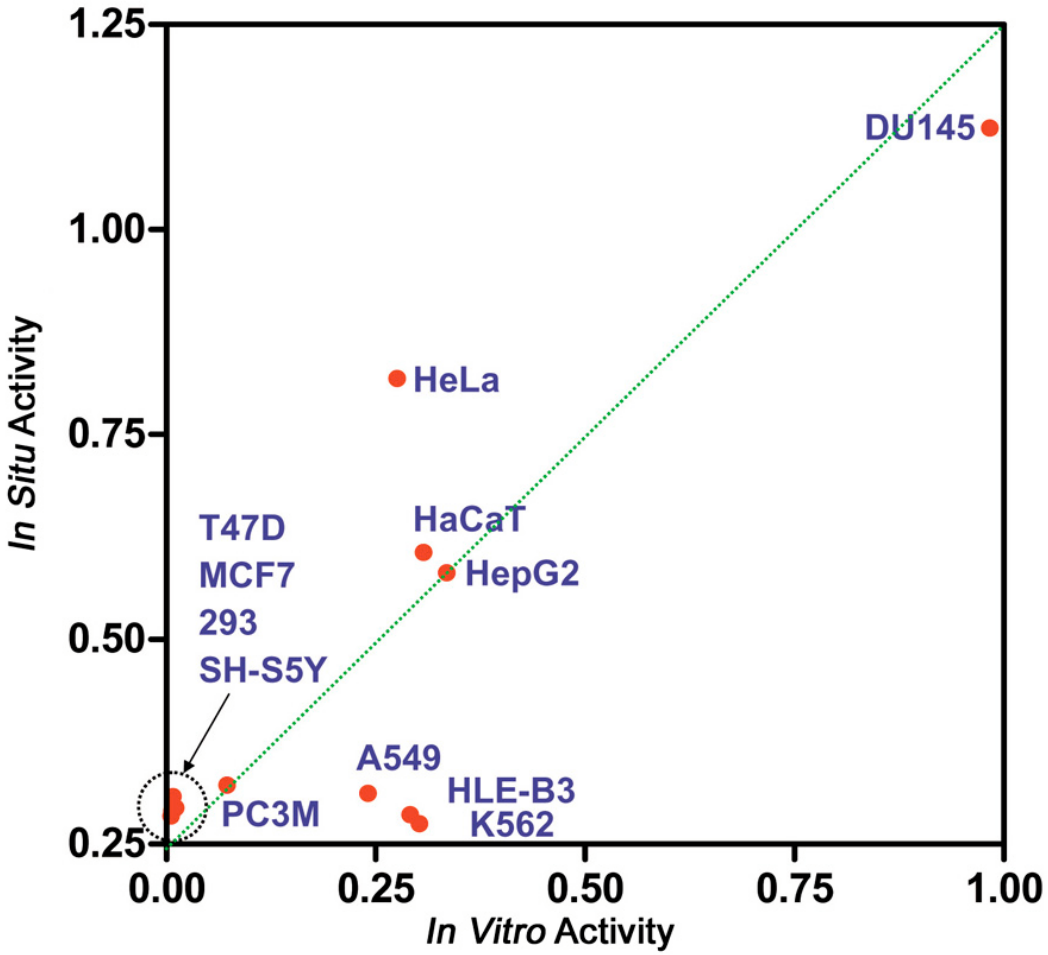

Figure 2. *In vitro* and *in situ* TG2 activity in various cell lines. The graph is reconstituted based on previous data (11). *In vitro* and *in situ* activities are expressed as radioactivity values expressed in cpm/h/mg ($\times 10^6$) and as the absorbance at 490 nm, respectively.

neuroblastoma cells treated with retinoic acid, protein levels of TG2 correlated well with *in vitro* activity, but not with *in situ* activity under the same conditions. *In situ* activity of TG2 increased only when calcium homeostasis was disrupted (9). Also, TG2 over-expression per se was not accompanied by increased *in situ* TG2 activity (10). Analysis of *in vitro* and *in situ* activities of TG2 in cell lines demonstrated no overall correlation between these two activities (Figure 2) (11). Thus, the quantitative increase of TG2 expression per se does not imply the increase of physiological activity in intact cells.

3.3. Intracellular TG2 is activated by oxidative stress

Transamidation activity of purified TG2 is activated by calcium and inhibited by GTP (2). However, the molecular mechanism by which intracellular TG2 is regulated is not known. Although it has been shown that proteolysis of the N-terminal activation domain (TG1 and factor XIIIa) or between the catalytic and barrel 1 domains (TG3) activates the intracellular enzymes (1), there is no evidence that proteolysis or other modification of the enzyme, such as phosphorylation, is involved in the regulation of TG2. Recently, it was demonstrated that intracellular TG2 is activated by endogenously generated reactive oxygen species (12) or exogenously administrated hydrogen peroxide (13). Because oxidative stress does not cause de novo synthesis of TG2 (13), these observations indicate the activation of the enzyme already existing in cells, probably due to calcium increase by oxidative stress. Thus, it is clear that activation by oxidative stress in intact cells is an important feature of TG2 which contributes to cellular response to viral infection as discussed in the following sections.

3.4. Protein-specific functional alterations of modified proteins

To precisely understand the roles of TG2 in virus-induced pathogenesis, the consequences of TG2 activation should be interpreted by the results obtained from functional analysis of the modified proteins. One aspect of the protein modifications catalyzed by TG2 is the variability in function resulting from the modifications, which is specific for each target protein. Polyaminated Tau and Rb, for example, are resistant to proteolysis by calpain

Table 1. Viral proteins modified by TG2

Viral proteins	Functions	Possible reactive residues	Related diseases
HPV 18E7	Oncoprotein (cell cycle deregulation)	Glu-87, Glu-88[1]	Cervical cancer, head and neck cancer
HIV gp120	Virus entry (binding to CD4 and chemokine co-receptor)	Glu-265[2]	Acquired immunodeficiency syndrome
HIV gp41	Virus entry (host-virus fusion)	Glu-51, Glu-52, Glu-66, Lys-77[2]	
HIV aspartyl protease	Virus maturation (Gag and Pol processing)	N.D.	
HCV core protein	Major capsid protein; RNA package	N-terminal 1-115 amino acids[3]	Hepatitis, liver cirrhosis, hepatocellular carcinoma

[1] Reactive glutamine or lysine residues for TG2 were determined by site-directed mutagenesis, [2] a series of peptide fragment analysis or [3] deletion mutant experiments. N.D.: not determined.

and caspase-7, respectively (14-15). Polyamination of phospholipase A_2 (PLA_2) was reported to increase its enzyme activity (16). Similarly, RhoA polyaminated by TG2 in cell culture causes increased RhoA activity (17). Even more interesting, polyamination affects protein-protein interactions, probably due to additional positive charges conferred by polyamines (18). Thus, understanding the nature of the altered protein-specific function and the type of modification that caused it can provide clues to the specific role of TG2 in the particular viral diseases.

4. TG2 AND VIRAL INFECTION

4.1. Mechanism of TG2 activation in virus-infected cells

As discussed above, it is apparent that intracellular TG2 is inactive under physiological condition. An emerging evidence suggests that TG2 is activated by viral infections through several mechanisms.

Role of calcium. A number of viruses including human immunodeficiency virus (HIV) and hepatitis C virus (HCV) increase intracellular calcium through expression of viral proteins, such as nonstructural protein 5A (NS5A) and NS3 encoded by HCV, and Nef and Tat by HIV (19-22). Also, oxidative stress caused by virus-mediated dysregulation of cellular antioxidant system, or by respiratory burst in phagocytes or neutrophils, or by pro-oxidant cytokines, such as TNF and IL-1, in response to viral infections also increases calcium concentration (23-30). Thus, increased calcium appears to be of central importance in the activation of TG2, although the mechanisms are not clear.

Role of interferons. Interferons (IFNs), antiviral cytokines, have been shown to modulate TG2 activity. IFN-alpha increases TG2 expression and activity (31). Increase of TG2 expression by INF-alpha is not due to induction of de novo synthesis, but due to inhibition of ubiquitination-dependent proteasomal degradation. INF-gamma also increases TG2 expression and activity (32). Unlike IFN-alpha, the increase in TG2 caused by INF-gamma is due to elevation of transcriptional activity (32). Increased transglutaminase activity has been reported in jejunal mucosa in patients with celiac disease in which TG2 has been identified as an autoantigen (33-34). The observation that IFN-gamma is expressed abundantly in the small intestine of patients with celiac disease suggests a possible role for IFN-gamma in the altered TG2 activity during viral infections (35).

Role of viral proteins. Interactions between viral proteins and host receptors can trigger signaling pathway(s) involved in TG2 induction. Binding of HIV envelope glycoprotein gp120 to CD4 receptor was shown to induce TG2 expression in human T cell clones (36). This *in vitro* finding was verified clinically; the expression of TG2 is far higher in peripheral blood mononuclear cells and lymph nodes from HIV-infected patients than in those from seronegative donors (37-38). Also, the plasma concentration of N [epsilon]-(L-gamma-glutamyl)-L-lysine isopeptide is increased in HIV-infected patients (39), indicating that the induced TG2 is functionally active. The mechanisms by which TG2 is induced are largely unknown.

4.2. Viral proteins modified by TG2

TG2 can catalyze post-translational modifications of exogenous proteins like viral proteins, as well as of cellular proteins. Table 1 summarizes the viral proteins modified by TG2.

HPV 18 E7 protein. HPV E7 is a major oncoprotein of high-risk HPV types, such as HPV 16 and 18, that inhibits tumor suppressor Rb as well as a number of cellular proteins leading to either abrogation of cell cycle control or induction of chromosomal abnormality in the development of cervical cancer (40-41). HPV 18 E7 was identified as TG2-interacting protein in HeLa cells (18). The conserved glutamine residue (Glu-87 and/or Glu-88) in the zinc binding domain of HPV 18 E7 is polyaminated by TG2. The modified HPV 18 E7 is unable to interact with Rb. Interestingly, E7 encoded by HPV 16, the most frequently detected type in cervical cancer (42), is not affected by TG2, because it lacks glutamine residues at the site where polyamination takes place in HPV 18 E7. This explains the high prevalence of HPV16 in cervical cancer.

HIV envelope proteins. Envelope glycoproteins of HIV are synthesized as a precursor (gp160) that undergoes proteolysis by cellular endoproteases, generating a surface protein gp120 and a transmembrane protein gp41 (43). The interaction of gp120 with CD4 and chemokine co-receptor triggers a conformational change in gp41, which allows HIV entry into target cells (44). TG2 catalyzes the modification of gp120 and gp41, but not of gp160 (45-46). However, the functional consequence or the physiological relevance of the HIV envelope proteins modified by TG2 is not known. Both gp120 and gp41 are promising targets for the development of inhibitors of viral entry (47). In fact, enfuvirtide (DP178 or T-20), a 36-amino-acid peptide derived from gp41, is the first FDA-approved drug that targets viral entry (48). Thus, exploring the role of TG2 in HIV entry may provide novel therapeutic rationales or strategies.

Table 2. TG2 substrates and their interactions with viral proteins

TG2 substrates	TG2 substrate-virus interactions
eIF5A	Interaction with HIV Rev
Rb	Interaction with HPV E7, adenovirus E1A, and SV40 large T antigen
IGFBP-3	Interaction with HPV E7
GAPDH	Binding to HCV, HAV, and human parainfluenza virus RNAs
HSP27	Interaction with HCV NS5A protein and HIV Vpr
HSP60	Interaction with HIV integrase, HIV gp41, HBV X protein, and HBV polymerase
HSP70	Interaction with Rotavirus VP5, adenovirus fiber protein, enterovirus capsid precursor P1, polyomavirus capsid proteins, vaccinia virus proteins, HPV L2 and E1, HBV envelope protein, HIV Vpr, HIV Gag, HTLV Tax and envelope protein, SV40 large T antigen, and Epstein-Barr virus nuclear antigen leader protein
HSP90	Interaction with HBV polymerase, influenza virus RNA polymerase, and reovirus attachment protein final sigma1
RhoA	Interaction with RSV fusion glycoprotein

HIV aspartyl protease. HIV aspartyl protease mediates the generation of Gag and Gag-Pol precursors (49). Chemical identification and isopeptide quantification provide evidence that HIV aspartyl protease can act as acyl donor as well as acyl acceptor substrate for TG2 (50). However, little is known about the alteration of HIV aspartyl protease activity caused by TG2- mediated modification. A number of peptidomimetic drugs, such as saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, fosamprenavir calcium, lopinavir, and atazanavir, are currently used for inhibiting HIV aspartyl protease (51). Thus, defining the role of TG2 in HIV maturation might be important in clinical and pharmaceutical aspects.

HCV core protein. HCV core protein, a major capsid protein that packages viral genomic RNA, regulates a number of cellular functions including signaling pathways, gene expression, cell growth and survival (52). HCV core protein is an efficient substrate for TG2 *in vitro* (53). In hepatocellular carcinoma, HCV core protein is cross-linked by treatment with calphostin C and serum deprivation, which are known to increase TG2 activity (53). This reaction is HCV-specific, as HBV core protein is not cross-linked by TG2, although the possibility that HBV core protein is polyaminated by TG2 can not be excluded. Cross-linked HCV core protein has a reduced ability to bind RNA. Thus, TG2 action may help the host to suppress HCV replication or RNA release in the early stage of infection.

4.3. Cellular proteins modified by TG2 during viral infection

Viral replication cycle encompasses a series of protein-protein interactions between the host and the virus. TG2 also modifies cellular proteins that interact with viral proteins and therefore affects host-virus interactions. Table 2 summarizes cellular proteins modified by TG2 during viral infection.

Eucaryotic initiation factor 5A. Eucaryotic initiation factor 5A (eIF-5A) is a cellular cofactor required for HIV-1 Rev, an essential viral protein for the nuclear export of viral RNA to the cytoplasm (54). eIF-5A is upregulated in HIV-infected peripheral blood mononuclear cells (55). In addition, eIF-5A is the only protein that contains hypusine formed by deoxyhypusine synthase and deoxyhypusine hydroxylase, and the inhibition of these enzymes suppresses HIV replication (56-57). eIF-5A expression is also increased by Epstein-Barr virus (EBV) nuclear antigen 2, a viral transcription factor that regulates viral and cellular genes, including the proto-oncogene c-myc (58). Thus, eIF-5A plays an important role in HIV replication and in the EBV-related diseases such as Burkitt's lymphoma. TG2 interacts stably with eIF-5A and modifies eIF-5A in the cells treated with calcium, magnesium and retinoic acid (59-60). However, the physiological significance of eIF-5A modification is unknown. Interestingly, the site of modification on eIF-5A is hypusine-50, and thus TG2 converts hypusine to gamma-glutamyl-omega-hypusine. Hypusine of eIF-5A is a critical residue for HIV RNA trafficking in coordination with HIV Rev (54). Therefore, it is probable that TG2 may interfere with the interaction between eIF-5A and HIV Rev or sequester eIF-5A from HIV Rev by stable interaction. In HIV-infected T cell, upregulated TG2 could modulate HIV replication through altering eIF-5A activity (37).

Retinoblastoma gene product. Tumor suppressor Rb is a target of several viral proteins, such as HPV E7, adenovirus E1A, simian virus large T antigen, and human cytomegalovirus IE2 86 (61-62). Rb has been shown to be a substrate for TG2, and modified Rb is resistant to degradation by caspase (15).

Insulin-like growth factor-binding protein-3. Insulin-like growth factor-binding protein-3 (IGFBP-3) has been implicated in apoptotic induction in several cell types (63). IGFBP-3 interacts with HPV E7, which inhibits apoptosis through proteasomal degradation of IGFBP-3. Recently, the apoptotic function of IGFBP-3 has been shown to be modulated by phosphorylation catalyzed by TG2 (64-65). Although intracellular kinase activity of TG2 is not verified yet, IGFBP-3 phosphorylation may alter the interaction between E7 and IGFBP-3. Thus, together with Rb modification, TG2 may interfere with the E7 function in several ways.

Glyceraldehyde-3-phosphate dehydrogenase. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) interacts with RNAs from human parainfluenza virus (66), hepatitis A virus (67) and hepatitis C virus (68). Its abundance in the cytoplasm and RNA helix-destabilizing activity suggests that GAPDH can directly influence viral translation and replication (68). GAPDH acts as acyl donor and acyl acceptor of TG2 (69-70). However, the potential role of TG2 in modulating virus-associated activity of GAPDH needs to be further investigated.

Heat shock proteins. Heat shock proteins (HSPs), a family of molecular chaperones that influence protein folding, have been known to interact with a variety of viral proteins (71). Virus-HSP interactions contribute to survival, apoptosis, and transformation of infected cells (71), and the development of innate or adaptive immune responses against viruses (72-73). HSPs have been shown to be substrates for TG2 *in vitro* as well as *in situ* (74-75). Although the functional consequences of HSP modifications are not well understood, TG2 may influence ATP-dependant chaperone activity of HSPs or the interaction between HSPs and viral proteins.

RhoA. RhoA is a Ras-related small G-protein that mediates a wide range of cellular processes, including cell morphology, motility, survival, and apoptosis (76). RhoA interacts with Tax protein of HTLV (77). Since Tax regulates viral and cellular transcription, Tax-RhoA interaction may explain the tumorigenesis of HTLV. RhoA also interacts with fusion (F) glycoprotein of respiratory syncytial virus whose infection causes acute lower respiratory tract illness in infants and young children (78), implicating in virus-mediated syncytium formation. RhoA is modified by cytotoxic necrotizing factor 1 (CNF1) toxin, a virulence factor produced by uropathogenic Escherichia coli. CNF1 deaminates Glu-63 of RhoA or catalyzes the transamidation of RhoA, leading to its activation and subsequent stress fiber formation (79-82). It has been demonstrated that TG2 also modifies RhoA to form stress fibers and focal adhesion complexes (17). Whether TG2 may play a role in morphological changes induced by viral infection requires a further study.

Cytoskeletal proteins. Actin and microtubule networks are involved in transport of virions that belong to herpesviridae, adenoviridae, rarvoviridae, poxviridae, from the cell surface to the sites of viral transcription and replication (83). Cytoskeletal proteins are modified by TG2, suggesting a possible role for TG2 in viral transport (84-86) via direct modification of cytoskeletal proteins or by RhoA-mediated rearrangement of the cytoskeleton network. The effect of TG2 on cytoskeletal functions may be a factor to be considered in the design of viral vectors and development of antivirals.

5. IMPLICATION IN HUMAN VIRAL DISEASES; IS TGASE 2 ACTIVATION A DOUBLE-EDGED SWORD?

Viral infections elicit host responses and viral virulence is attributable to the outcome of virus-host interactions. Thus, defining virus-host interactions is important for understanding viral pathogenesis and associated cellular processes. Furthermore, because both viral and host proteins could be promising targets, understanding the nature of their interactions can facilitate the development of novel antiviral strategies (87).

The observation that TG2 is up-regulated and/or activated through inflammatory and oxidative stress responses induced by viral infection suggests that TG2 may act as an anti-viral host factor. Viral proteins associated with entry (HIV), replication (HPV) and packaging (HCV) in virus-infected cells are cross-linked or polyaminated in the processes mediated by TG2 (18, 45-46, 50, 53). The question of how TG2-mediated modifications affect the life cycle of virus is difficult to answer due to a lack of appropriate animal or cell culture models that overcome species-specificity of viral infection. Although no experimental evidence that directly addresses this question is yet available, emerging *in vitro* data indicate that TG2 alters the physicochemical nature of the viral proteins and thereby influences their activity (18, 53). The fact that TG2 activators that are used in the clinical situations, such as mycophenolic acid, suppress HCV replication (88-90), underscores the physiological importance of this physicochemical modification. Another possible way for TG2 to inhibit the viral proteins is to alter protein-protein interactions through the modification of cellular proteins, as demonstrated with eIF-5A (54, 60). TG2 may interact stably with viral or cellular proteins and prevent the formation of virus-host protein complexes as previously suggested (59). Thus, TG2 may play an important role in controling virus infections and thus viral disease progression.

An important related question is whether over-expression or over-activation of TG2 in response to persistent viral infection can predispose some individuals to virus-related pathology such as fibrosis or malignant transformation. TG2 is implicated in a wide range of biological processes involving modification of cellular proteins at various locations, including apoptosis, cell adhesion, differentiation and formation of the extracellular matrix (2, 5). Experimental and clinical studies have shown that aberrant TG2 activity is involved in the pathogenesis of cataract (13), CAG-expansion diseases (91), celiac disease (92), cardiac hypertrophy (93), and type-2 diabetes (94). Thus, one might hypothesize that sustained TG2 activation caused by persistent infection may have pathological consequences. This hypothesis is strongly supported by the finding that TG2 expression correlates with the stage of fibrosis in HCV-infected livers (95-96). In addition, TG2 activation elicits prolonged inflammatory responses through PLA2 or COX-2 activation (16, 93, 97-98). In this regard, one might see the pathological consequences of viral infection as an undesirable byproduct of the antiviral functions of TGase 2. Based on available direct as well as circumstantial evidence, we propose a model for the physiological and pathological roles of TG2 during viral infection (Figure 3).

In addition to transamidation activity, TG2 is known to have another function such as GTP hydrolyzing activity that might also contribute to its antiviral activity. Identification of other viral or cellular substrates for TG2, characterization of other types of protein modifications brought on by TG2 and elucidation of molecular mechanisms leading to aberrant

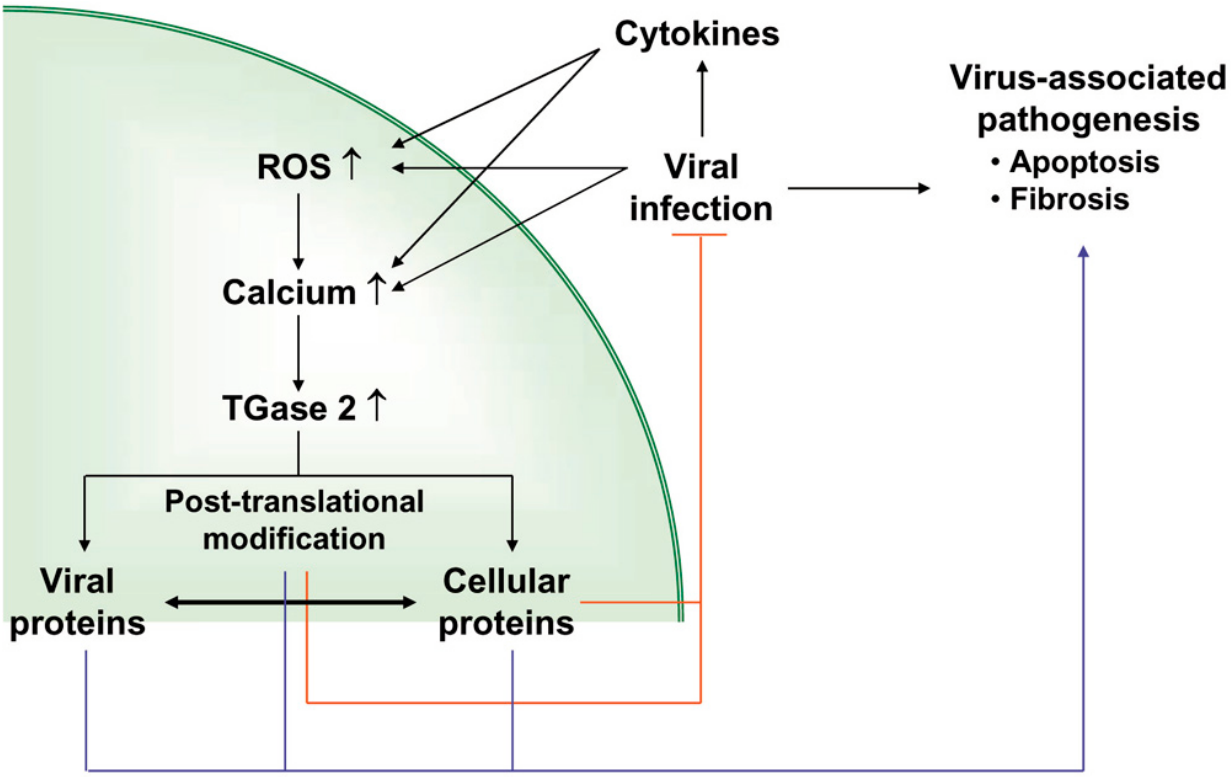

Figure 3. A model for the physiological and pathological roles of TG2 during viral infections. Viral infection and host responses induce oxidative stress and disturb intracellular calcium homeostasis, resulting in activation of TG2. TG2-mediated modification of both viral and cellular proteins interferes with the activity of viral proteins and/or protein-protein interactions that could affect the viral life cycle. On the other hand, persistent infection may result in sustained TG2 activation, leading to pathological consequences.

TG2 activation should lead to novel strategies in the therapy of viral disorders.

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Abbreviations: TG2: transglutaminase 2; HPV: human pappilomavirus; HBV: hepatitis B virus; HCV: hepatitis C virus; HIV: human immunodeficiency virus; IFN: interferon; eIF: eukaryotic initiation factor; IGFBP: insulin-like growth factor binding factor; GAPDH: glyceraldehydes-3-phosphate dehydrogenase; HSP: heat shock protein

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