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Review

Regulation of a Novel Guanine Nucleotide Binding Protein Tissue Transglutaminase ($G\alpha_h$).

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Tissue transglutaminase (TGII, Gαh) belongs to a family of enzymes which catalyze post-translational modification of proteins by forming isopeptides via Ca^{2+}-dependent reaction. Although TGII-mediated formation of isopeptides has been implicated to play a role in a variety of cellular processes, the physiological function of TGII remains unclear. In addition to this TGase activity, TGII is a guanosine triphosphatase (GTPase) which binds and hydrolyzes GTP. It is now well recognized that the GTPase action of TGII regulates a receptor-mediated transmembrane signaling, functioning as a signal transducer of the receptor. This TGII function signifies that TGII is a new class of GTP-binding regulatory protein (G-protein) that differs from "Classical" heterotrimeric G-proteins. Regulation of enzyme is an important biological process for maintaining cell integrity. This review summarizes the recent development in regulation of TGII that may help for the better understanding of this unique enzyme. Since activation and inactivation of GTPase of TGII are similar to the heterotrimeric G-proteins, the regulation of heterotrimeric G-protein in the transmembrane signaling is also discussed.

Keywords: Transglutaminase II, Gah, Regulation, Expression, GTPase, TGase.

Regulation of TGII functions in transmembrane signaling

Transglutaminases (TGases) are a family of enzymes that catalyze the post-translational modification of proteins by formation of ε-(γ-glutamyl)lysine isopeptide bond between or within proteins (Folk, 1980; Greenberg *et al.*, 1991). There are six transglutaminases, including keratinocyte (TGase I), tissue (TGase II), epidermal (TGase III), plasma (factor XIIIa), prostate (TGase IV), and TGase X transglutaminases (Chen and Mehta, 1999). Among these TGases, tissue transglutaminase (TGII, $G\alpha_h$) has an extra enzyme activity GTPase that binds GTP and hydrolyzes it to GDP and phosphate (Pi) (Im *et al.*, 1997). TGII is a ubiquitous enzyme and expressed highly in certain cells and tissues such as liver and heart. Physiological function of TGase of TGII remains unclear, and regulation of isopeptides is completely unknown. The isopeptide-specific protease(s) has not been identified. However, irreversible isopeptide formation of various proteins has been observed in diverse cellular processes, including cell differentiation, cell adhesion, and induction of apoptosis (for detailed information on physiological role of TGII, see refs., Melino and Piacentini, 1998; Chen and Mehta, 1999).

In a variety of signaling pathways, heterotrimeric G-proteins consisting of 39-46 kDa α (GTPase, Gα) 36-37 kDa β (Gβ), and 5-7 kDa γ (Gγ) subunits mediate cell surface receptor signals to various effectors (Gilman, 1989; Dohlman *et al.*, 1991; Im, 1996; Im *et al.*, 1997). There are over 1000 types of receptors, which share a characteristic topological structure, having seven α-helical transmembrane spanning domains. These transmembrane segments are interconnected by three extracellular and intracellular loops. The stimulated receptors by the binding of hormones, neurotransmitters, and sensory stimuli, induce GDP/GTP exchange of their cognate G-proteins. To date, 17 Gα, 9 Gβ, and 9 Gγ subunits of heterotrimeric G-proteins have been identified and characterized for their specific functions. To amplify a single ligand signal, one ligand-bound receptor interacts with a large number of one specific G-protein and/or multiple G-proteins. The interaction process sustains until the receptor is desensitized by phosphorylation. The GTP-bound Gα dissociates from Gβγ subunits. It should be noted that Gβγ subunits do not dissociate from each other in native state. Both Gα and Gβγ dimers are involved in regulation of effector

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activity. In most of cases, these free forms of subunits of G-protein activate various effectors such as adenylyl cyclase, cGMP phosphodiesterase, and phospholipase C (PLC) but also inhibit effectors; the βγ subunits and GTP-bound $G\alpha_i$ inhibit adenylyl cyclase (Sunahara *et al.*, 1996; Rhee and Bae, 1997). The effectors generate the second messengers, including cAMP, inositol 1,4,5-triphosphate (IP_3), diacylglycerol (DAG), and cGMP. These second messengers induce dramatic cellular changes, activation of kinases, gene transcription, cytoskeleton reorganization, secretion, and membrane depolarization. To terminate transmembrane signaling, the active forms of the subunits of G-proteins are deactivated when GTP is hydrolyzed by Gα, thereby reforming heterotrimers. Thus, G-proteins are known as molecular switches in onset and offset of signaling. In this termination step of the signal, it is now recognized that there exist a large number of GTPase-activating proteins (GAPs) for Gα proteins known as regulators of G-protein signaling (RGS) (Berman and Gilman, 1998; DeVaries and Gist Farquhar, 1999). Independent from action of these RGS proteins, certain effectors in the G-protein-coupled receptor system regulate the signaling pathways by acting as a GAP or guanine nucleotide-exchanging factor (GEF) on the cognate Gα proteins (Arshvsky *et al.*, 1992; Bernstein *et al.*, 1992; Scholich *et al.*, 1999). For example, PLCβ1 and the γ-subunit of cGMP phosphodiesterase directly accelerate GTP hydrolysis by $G\alpha_q$ (Bernstein *et al.*, 1992) and $G\alpha_t$ (Arshvsky *et al.*, 1992), respectively. Adenylyl cyclase facilitates GTP binding and GTP hydrolysis by the cognate G-protein G_s, functioning as both GEF and GAP (Scholich *et al.*, 1999). These findings indicate that the effector also modulates its cognate Gα (GTPase function) to terminate or facilitate the signal (control of the second messenger level).

The first piece of evidence that TGII is a GTPase and acts as a signal transducer in receptor-mediated transmembrane pathway is co-isolation of a ternary complex containing TGII (termed G_h) and ligand-bound α_{1B}-adrenoreceptor (α_{1B}-AR) from rat liver (Im and Graham, 1990; Im *et al.*, 1990). Since then, significant progress has been made in the field of TGII-mediated transmembrane signaling and its regulation. Numbers of the TGII-coupled receptors are increasing. The receptors are the $\alpha_{1B/D}$-AR (Im and Graham, 1990; Nakaoka *et al.*, 1994; Chen *et al.*, 1996; Feng *et al.*, 1999a; Wu *et al.*, 2000), thromboxane A_2 (Vezza *et al.*, 1999) and oxytocin (Park *et al.*, 1998) receptors. It also appears that TGII selectively interacts with these receptors in a subtype-specific manner (Chen *et al.*, 1996; Vezza *et al.*, 1999). The specific coupling ability of TGII suggests that physiological responses mediated by TGII differs from those mediated by G_q, although these G-proteins stimulate PLCs. Most of the receptors, which couple with bacterial toxin (cholera and/or pertussis toxin)-insensitive G-proteins, are shown to interact with G_q and its family of proteins and stimulate PLC-β. TGII-involved transmembrane signaling pathway is shown in Figure 1. TGII is associated with a 50 kDa protein ($G\beta_h$) (Im *et al.*, 1990)

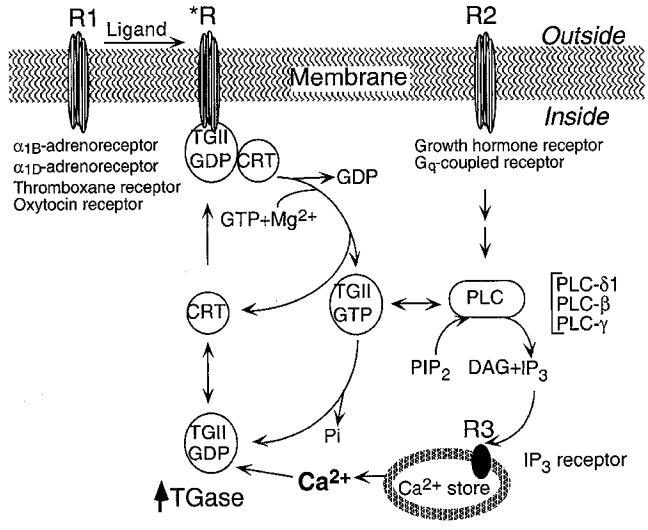

Fig. 1. The GTPase cycle of TGII-CRT complex (G_h) involving the cognate receptors and effector. The TGII-coupled receptors recruit G_h (GDP-TGII-CRT complex) upon binding of ligand. The interaction of GDP-bound TGII-CRT with the ligand-bound receptor promotes GTP binding to TGII, followed by GDP release. GTP-bound TGII dissociates from CRT and interacts with PLC-δ1, which hydrolyzes phosphatidyl 4,5-biphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). The TGase activity of GDP-bound TGII can be stimulated by increasing of intracellular calcium. The calcium level is increased by the activation of PLCs, followed by activation of calcium-mobilizing receptors (see refs Feng *et al.*, 1999b; Zhang *et al.*, 1998).

which is known as a calcium binding protein calreticulin (CRT, see ref. Feng *et al.*, 1999b). Thus, G_h (TGII-CRT complex) is a heterodimer consisting of 70-80 kDa TGII and 50 kDa CRT. The ligand-bound receptor (activated form) interacts with GDP-bound TGII-CRT complex with high affinity: the complex can be isolated using wheat-germ agglutinin or specific ligand cross-linked resin (Im and Graham, 1990; Baek *et al.*, 1993; Park *et al.*, 1998). The interaction induces a conformation of TGII that has a low affinity for GDP, thereby allowing TGII to release GDP and bind GTP (Im *et al.*, 1990; Baek *et al.*, 1996). Once GTP binds to the TGII-CRT complex, GTP-bound TGII dissociates from CRT: GTP-bound TGII is not able to be coimmunoprecipitated with CRT, while GDP-bound TGII does (Baek *et al.*, 1996; Feng *et al.*, 1999b). The GTP-TGII interacts with effectors: PLC-δ1 in the presence of GTP (Feng *et al.*, 1996; Park *et al.*, 1997). The intrinsic GTPase reaction of TGII which hydrolyzes GTP to GDP and Pi, terminates stimulation of the effectors, and subsequent association of GDP-bound TGII with CRT completes one cycle of the transmembrane signaling. Although we have previously postulated that TGII exists as TGase form (Nakaoka *et al.*, 1994), recent studies indicate that TGII in cell exists as

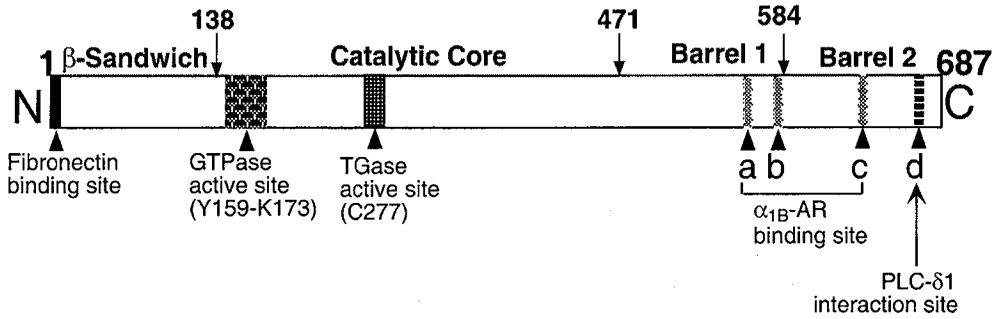

Fig. 2. Schematic presentation of various binding and functional domains in TGII. Active site of TGase contains a conserved motif consisting of YGQCWVF. Cysteins 277 residue is critical for the enzyme activity. Segments a, b, and c denote the three regions L^{547}-L^{561}, R^{564}-^{581}, Q^{633}-E^{646}, respectively. Segment denotes the region V^{665}-K^{672}.

guanine nucleotide-bound form (Zhang *et al.*, 1998). Our recent studies also demonstrate that GDP-bound TGII exhibits TGase activity in increased Ca^{2+} concentrations and that Ca^{2+}-mediated TGase stimulation of GDP-TGII is inhibited by CRT (Feng *et al.*, 1999b). On the basis of these observations, we postulate that GDP-bound TGII acts as TGase in cell.

Regulation of PLC-δ1 by GTP-TGII is the change of the affinity of PLC-δ1 for Ca^{2+} with no increase in the turnover. The PLC activity also exhibits a biphasic response to the calcium concentration and occupancy of guanine nucleotide by TGII (Das *et al.*, 1993). Thus, the enzyme activity was stimulated with low concentrations of Ca^{2+} (≤10 μM in vitro reconstitution) by GTP-bound TGII, whereas the enzyme activity was subsequently inhibited when concentrations of Ca^{2+} were increased. In contrast, in the presence or absence of GDP, the enzyme was stimulated with high concentrations of Ca^{2}+ (≤20 μM) where stimulation of the enzyme by GTP-TGII was inhibited. Similarly, Murthy *et al.* (1999) reported that GTP-TGII inhibited PLC-δ1, while GDP-TGII stimulated the enzyme. The Ca^{2+}dependency was not clearly defined in this study. The TGII-mediated PLC stimulation is also modulated by the level of TGII expression (Zhang *et al.*, 1999). At low levels of TGII expression, the α_{1B}-AR-mediated PLC activity was increased, whereas the receptor-mediated PLC stimulation was inhibited when TGII was highly expressed. These multiple regulations of PLC-δ1 by TGII have led to postulate two mechanisms: one is GTP-TGII-mediated stimulation; the other is GDP-TGII-mediated. In either way, our original observations, that TGII biphasically regulates the PLC activity, embrace both mechanisms. In addition, it is well known that the PLC-δ1 activity is also inhibited by IP_3, competing with its substrate PIP_2 for a binding site known as the pleckstrin homology (PH) domain (Cifuentes *et al.*, 1994v; Lemmon *et al.*, 1995). Studies have also demonstrated that an increase in the intracellular concentration of Ca^{2+} activates PLC-δ1 (Allen *et al.*, 1997; Kim *et al.*, 1999), indicating that activation of PLC-δ1 occurs secondarily in response to the receptor-mediated activation of other PLCs or Ca^{2+} channels. A GAP for the small GTPase RhoA (RhoGAP) also activates PLC-δ1 by direct association (Homma and Emori, 1995). All of these observations suggest that the PLC-δ1 activity is regulated by multiple factors. In addition, Banno *et al.* (1994) have demonstrated that the thrombin receptor activates PLC-δ1 in the presence of GTPγS a nonhydrolizable GTP analog. Since it is known that PLC-δ1 is not activated by heterotrimeric G-proteins, it is tempting to speculate that one subtype of thrombin receptors couples with TGII.

Functional domains of TGII

The human gene has been mapped on chromosome 20 (q12) and comprises 32.5 kb of 13 exons and 12 introns (Gentile *et al.*, 1994; Fraij and Gonzales, 1997). Analysis of guinea pig primary structure has revealed that, despite having 17 cysteine residues. However, this molecule contains no disulfide bond (Ikura *et al.*, 1988). Although TGII also contains 6 potential N-glycosylation consensus motifs, TGII is not glycosylated. Figure 2 shows schematic representation of various binding and functional domains in TGII. The active site of TGase is highly conserved among species and consists of YGQCWAF motif, and for the enzyme activity, cysteine residue located at 277 in human (Gentile *et al.*, 1991) and 276 guinea pig is critical (Ikura *et al.*, 1988). Substitution of this residue results in impairment of the TGase activity but not the GTPase activity (Lee *et al.*, 1993). TGII shows no homology with other GTP-binding proteins. This fact has raised a doubt whether TGII is a GTPase. Recently, Iissmaa *et al.* (2000) have mapped the GTP binding site. The GTP binding site locates to a 15-residue segment ^{159}YVLTQQGFIYQGSVK^{173} of TGII core domain that differs significantly from the other GTP binding proteins. TGII is thus a novel GTP-binding protein (GTPases). Two amino acid residues (Ser171 and Lys173) are critical for the binding and hydrolysis of GTP by TGII. Mutation of these two residues resulted in impairment of the GTPase action of TGII, GTP binding, hydrolysis, and signal transduction of the α_{1B}-AR. The impairment of the signal transduction of the mutants provides an important mechanism that the receptor signal to the effector by GTP-bound TGII, consistent with our original findings that

activation of the receptor promotes GTP binding to TGII (Im and Graham, 1990; Im *et al.*, 1990). The α_{1B}-AR interaction sites within TGII have been mapped by peptide and site-directed mutagenesis approaches (Feng *et al.*, 1999a). The regions L^{547}-I^{561}, R^{564}-D^{581}, and Q^{633}-E^{646} are the binding sites of the receptor in TGII. Among these interaction sites, R^{564}-D^{581} and Q^{633}-E^{646} are the high affinity binding sites for the receptor. The interaction site of TGII for PLC is very near the end of C-terminus PLC (Hwang *et al.*, 1995). A region of eight amino acid residues (V^{665}-K^{672}) is critical for recognition and stimulation of PLC as well as PLC-δ1 (Feng *et al.*, 1996; Feng *et al.*, 1999a). Fibronectin, which is an extracellular matrix protein and a ligand for certain members of integrin family proteins, binds to the very end of the N-terminus: a segment of seven amino acid residues (Ala2- Glu8) is the binding site (Jeong *et al.*, 1995). Fibronectin is also involved in externalization of TGII (Lorand *et al.*, 1988). Calcium is an activator for TGase activity of TGII. However, the exact calcium binding site has not been identified. Mutation of Glu residues among putative Ca^{2+} binding residues (Asn398, Asp400, Glu447, and Glu452 in human TGII) did not inhibit the TGase activity completely indicating the presence of a secondary Ca^{2+}-binding site (Ikura *et al*, 1995).

Regulation of TGII expression

Characterization of the TGII gene isolated from guinea pig (Suto *et al.*, 1993), human (Lu *et al.*, 1995), and mouse (Nagy *et al.*, 1997) has provided important information for understanding of regulatory mechanism of TGII expression. Analysis of the 5' upstream nucleotide sequences in the TGII gene has revealed that the TGII promotor contains a number of functional responsive elements and binding sites, indicating that TGII expression is regulated by multiple factors. The responsive elements include glucocorticoid, interleukin-6, AP1 and AP2. The core region of human TGII promotor contains TATA box, four AP1 sites, and four necrosis factotr-1 sites within 134 bp upstream of the translation start site. This core region was sufficient for accomplishing high constitutive transcriptional activity, and the four SP1 sites contribute to the high basal promotor activity (Lu *et al.*, 1995). The sequences corresponding to the retinoid responsive element are not identified in either guinea pig or human TGII promotors yet. However, *cis*-acting elements necessary for directing retinoid-dependent transactivation of mouse TGII are shown to locate within the proximal 3.8 kb DNA that flanks the 5' end of TGII gene (Nagy *et al.*, 1996). Detection analysis of the region has revealed that two critical retinoid-responsive sites locate within 1.7 kb upstream of the transcriptional start site (Nagy *et al.*, 1996). This region contains a triplicated retinoid receptor binding motif (mTGRRE1). Transgenic mice containing TGII promoter-β galactosidase reporter gene have shown a specific pattern of TGII transgene expression in cartilage, in cells of apical ectodermal ridge, and in interdigital mesenchime (Nagy *et al.*, 1997). Transient expression studies have shown that numerous cell lines exhibit high constitutive promotor activity (Lu and Davies, 1997). It also appears that methylation of GpC-rich region of human promotor inhibits the transcriptional activity, indicating that DNA methylation plays a role in regulating TGII expression. In addition, consistent with the data from the analysis of the TGII gene, many cytokines are shown to induce TGII expression. These cytokines are retinoic acid (Melino and Piacentini, 1998), interleukin-6 (Sato *et al.*, 1993), transforming growth factor-β (Kojima *et al.*, 1986) and tumor necrosis factor (Kuncio *et al.*, 1998).

Regulation of TGase and GTPase activities of TGII

TGII is constitutively expressed in endothelial and aortic smooth muscle cells, vein, capillaries, and other organ-specific cells such as mesengial, renonmedullary interstitial, and colonic pericryptal fibroblasts (Thomazy *et al.*, 1989; Baek *et al.*, 1993). It is well established that regulation of TGase activity of TGII requires Ca^{2+} and GTP and Ca^{2+}-bound TGII was unable to bind GTP, whereas GTP binding to the enzyme inhibits TGase activity (Achyuthan and Greenberg, 1987). In cells, concentration of GTP and calcium are ~100 μM and ~100 nM, respectively (Zhang *et al.*, 1998). If the physiological concentration of Ca^{2+} is sufficient to inhibit GTP binding to TGII, the TGase activity would readily be stimulated. However, under the physiological concentrations of GTP, TGII-mediated cross-linking activity was not observed (Smethurst and Griffin, 1996; Zhang *et al.*, 1998). If the physiological concentration of Ca^{2+} cannot inhibit the GTP binding, TGII can readily bind and hydrolyze GTP: TGII would constitutively activate PLC-δ1 or open ion channels without activation of receptor. Moreover, since GTP-bound TGII arrested G_2/M phase (Mian *et al.*, 1995), the enzyme can inhibit the cell growth. These findings clearly indicate that expression of TGII and/or the enzyme activity should be strictly regulated. Therefore, it is reasonable to postulate that there is a regulatory protein which maintains TGII in the inactive state. We have previously reported that TGII consistently copurified with a 50 kDa protein which inhibits GTP binding to TGII (Im *et al.*, 1990; Baek *et al.*, 1996). The identity of this protein has been revealed recently. $G\beta_h$ is the same protein known as calreticulin (CRT) a calcium-binding protein (Feng *et al.*, 1999b). Thus, CRT inhibited GTP binding to TGII and the TGase activity of GDP-bound TGII in an allosteric fashion. These findings demonstrate that the interaction site(s) of CRT in TGII is separated from the Ca^{2+} binding site. This protein interacted with GDP-TGII but not GTP-TGII and empty form of the GTPase active site, consistent with a report that TGII in cells exists as a guanine nucleotide-bound form (Zhang *et al.*, 1998). All of these observations indicate that GDP-TGII-CRT is the inert state of TGII in cells. In addition, sphingosylphosphocholine has been shown to activate TGase activity by reducing Ca^{2+} requirement (Lai *et al.*, 1997). Physiological relevance of this phospholipid

remains unclear.

Calreticulin is a calcium binding protein with high Ca^{2+} binding capacity and plays an important role in Ca^{2+} storage in endoplasmic/sarcoplasmic reticulum (Nash *et al.*, 1994). Supporting the interaction of CRT with TGII, studies have shown that CRT is found outside of these calcium store compartments. For example, immunoreactive CRT was detected in the nuclear envelope and the nucleus of certain cell types (Opas *et al.*, 1991), in the acrosome of sperm cells (Nakamura *et al.*, 1992), and cytolytic granules of T-cells (Dupuis *et al.*, 1993). The protein was also found in cytoplasm and plasma membrane (McDonnell *et al.*, 1996) and serum (Sueyoshi *et al.*, 1991). Structural analysis of CRT has revealed that the protein contains two calcium-binding domains, P (proline rich)- and C (C-terminal)-domains (Bakish *et al.*, 1991). The P-domain is the high-affinity Ca^{2+} binding site with low capacity (Kd, ~10 μM; binding capacity, 1 mol mol^{-1}), and the C-domain is the low-affinity Ca^{2+} binding site with a high capacity (Kd, ~250 μM; binding capacity 25 mol mol^{-1}). The C-domain is thus involved in Ca^{2+} storage. The finding of high-affinity Ca^{2+} binding site (P-domain) has been suggested to play a regulatory role (Baksh *et al.*, 1991; Nash *et al.*, 1994). Supporting the notion, numerous studies have demonstrated a regulatory role of CRT in signaling propagation of integrins and nuclear receptors. This regulatory role of CRT appears to be displayed in different ways dependent on its cellular localization. Cytoplasmic CRT acts as the positive regulator for integrins by interacting with cytoplasmic tail of the α-subunits of integrins (Leung-Hagesteijn *et al.*, 1994). The interaction is involved in integrin-mediated Ca^{2+} influx and cell adhesion (Coppolino *et al.*, 1997). In contrast, the nuclear CRT functions as a negative regulator by interacting with a conserved region in the DNA binding domain of various nuclear receptors, including retinoid receptor and glucocorticoid receptor (Burns *et al.*, 1994; Dedhar *et al.*, 1994). Therefore, it is tempting to speculate that TGII is also involved in these receptor-mediated cellular events.

Concluding Remarks

Cardiac specific overexpression of TGII in mice has resulted in mixed responses (Small *et al.*, 1999). Increased basal PLC stimulation was not observed. However, contractility of heart was reduced, accompanying in depressed fractional shortening, contraction, and relaxation. Fibril formation was observed interstitial of the cardiac cells, and contractile genes were upregulated in cardiac tissue. Although these results clearly indicate that TGII plays a role in cardiac pathogenesis, the mechanism remains unclear. To date, it is clear that TGII has two biological functions, GTPase and TGase activity. Regulation of this enzyme activity is probably a key to understanding the physiological function of TGII. GTP binding to TGII and level of Ca^{2+} are regulated by the external stimuli and TGII is associated with a negative regulator CRT. These facts strongly suggest that the physiological role of TGII correlates with the physiological role of TGII-coupled receptors as well as its cognate effectors. Although PLC-δ1 is an effector for TGII, it is likely that there are unidentified effectors. Until these effectors are identified, we may not clearly understand the physiological functions of this protein in a living system.

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