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Review

The emerging structural understanding of transglutaminase 3

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Abstract

Transglutaminases (TGase; protein-glutamine: amine γ-glutamyl-transferase) are a family of calcium-dependent acyl-transfer enzymes ubiquitously expressed in mammalian cells and responsible for catalyzing covalent cross-links between proteins or peptides. A series of recent crystal structures have revealed the overall architecture of TGase enzymes, and provided a deep look at their active site, calcium and magnesium ions, and the manner by which guanine nucleotides interact with this enzyme. These structures, backed with extensive biochemical studies, are providing new insights as to how access to the enzyme's active site may be gated through the coordinated changes in cellular calcium and magnesium concentrations and GTP/GDP. Calcium-activated TGase 3 can bind, hydrolyze, and is inhibited by GTP, despite lacking structural homology with other GTP binding proteins. A structure based sequence homology among the TGase enzyme family shows that these essential structural features are shared among other members of the TGase family. Published by Elsevier Inc.

Keywords: Transglutaminase 3; X-ray crystallography; Calcium ion; GTP binding

1. The biology of transglutaminase 3

The transglutaminase 3 (TGase 3) enzyme is expressed in many tissues including epithelia, brain, and placenta (Ahvazi et al., 2002a,b; Greenberg et al., 1991; Kim et al., 1990, 1999; Steinert and Marekov, 1995; Steinert et al., 1998). In the epidermis, its principal role is in cross-linking a number of structural proteins to form a cell envelope (CE) barrier structure essential for survival (Candi et al., 1999; Melino et al., 1998; Steinert et al., 1999). Sequential cross-linking of several integral components catalyzed by TGases leads to a gradual increase in the thickness of the CE and underscores its rigidity. The most abundant key structural players in this cross-linking process include loricrin, SPRs (small proline-rich proteins), and involucrin that are rich in lysine and glutamine and function primarily as CE components. The others include keratins, filaggrin, desmoplakin, and envoplakin that are keratin, kertain-associated, or desmosomal proteins. The desmosomal components include desmoplakin, plakoglobin, and desmoglein. The calcium-regulated proteins such as S100A11, SA100A10, annexins I and II, elafin, and cystatin A family proteins also have functions in growth and differentiation.

In the hair follicle, the TGase 3 is responsible for cross-linking trichohyalin and keratin intermediate filaments to harden the inner root sheath, which in turn is critically important for hair fiber morphogenesis (Lee et al., 1996). In the hair fiber central column of medulla cells, it also cross-links trichohyalin to itself to form a vacuolated structure that entraps pockets of air, and is thereby of importance for maintenance of body heat in mammals (Steinert et al., 2003). By RT-PCR and indirect immunofluorescence methods, the TGase 3 enzyme has been demonstrated as being expressed in certain compartments of normal brain (Hitomi et al., 2001). However, its natural substrates in this tissue is not yet known. Furthermore, TGase 3 is markedly upregulated in a variety of degenerative diseases, with the

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implication being that it participates in the aberrant cross-linking of cell proteins that exacerbate disease progression (Kim et al., 2002). Moreover, one report has demonstrated the occurrence of TGase 3 autoantibodies in patients with dermatitis herpatiformis (Kim et al., 1999). Attempts to generate a knockout model for TGase 3 in mice have met with failure, as there is no implantation, and therefore no embryo for examination. Thus, it appears that the TGase 3 enzyme is essential for the earliest stages of development. It remains possible that there is, nevertheless, no disease associated with seriously depleted TGase 3 activity in adults (Fesus and Piacentini, 2002; Lorand and Graham, 2003). In this regard, the TGase 3 enzyme may be very different from the TGase 1 system, in which deactivating mutations allow for near-normal fetal development, but are inimical to later life because of catastrophic water barrier function defects. The importance of TGase enzymes in CE formation is dramatically illustrated by lamellar ichthyosis, a severe skin disease that results from loss of function mutations in TGase 1. In that case, other TGase isoforms can replace the lost protein cross-linking functions of TGase 1 but cannot replace the membrane-bound function of ceramide ester attachment for water barrier function (Nemes et al., 1999, 2000; Steinert et al., 1996). It remains to be seen whether other TGase isoforms can replace the lost protein cross-linking function of TGase 3 (Tarcsa et al., 1998). Moreover, TGases may have other functions unrelated to protein cross-linking. For instance, it has been shown that the TGase 2 is able to function as PDI (protein disulphide isomerase, Hasegawa et al., 2003).

Here, we summarize the new structural/functional information on the TGases, with particular emphasis on the TGase 3 enzyme system for which several new crystallographic analyses are now available (Table 1). These studies provide snapshots of TGase 3 switching between its off state when bound to Mg^{2+}/GTPγS, and its on state when bound to Ca^{2+}/GDP. A structure-based sequence analysis shows that the major new functional lessons which have been uncovered for TGase 3 are shared with other TGase family members (Fig. 1).

2. The activation of TGase 3

The TGase 3 enzyme is initially expressed as an inactive 77 kDa soluble pro-enzyme consisting of 692 amino acids and requires proteolysis to become active in transamidation reactions. The proteolysis involves cleavage of the enzyme at a specific site yielding two globular domains, a 50 kDa amino terminus that contains catalytic sites and a 27 kDa carboxy terminus fragment that remain held together (Kim et al., 1990, 1993). The cleavage site is located on a long flexible loop that connects the catalytic core domain and the β-barrel 1 domain. The size and sequence of this loop is unique to TGase 3; interestingly, cleavage at similar sites in tissue transglutaminase 2 (TGase 2) and human factor XIIIa (fXIIIa) enzymes deactivates these enzymes. The nature of the natural proteases used for cleavage in vivo is not known, but this process can be mimicked in vitro using dispase protease (Kim et al., 1993, 2001).

3. Architectural design of the TGase 3 active site

Active TGases catalyze a posttranslational modification linking low molecular weight amines to proteins, or induce an isopeptide bond between or within polypeptide chains leading to a cross-linking that stabilizes a macromolecular protein network. Previously, the structures of the fXIIIa (Weiss et al., 1998; Yee et al., 1994), and a fish TGase enzyme (fTG, equivalent to mammalian TGase2, Noguchi et al., 2001), provided the only models for the TGase enzymes. A recent paper reported the X-ray structures of the zymogen (with one Ca^{2+} ion) and active (with three Ca^{2+} ions) forms of TGase 3 (Ahvazi et al., 2002a,b, 2003) that exists as monomer in solution (Kim et al., 1990). All of these proteins have similar domains: the amino terminal β-sandwich domain; the catalytic core domain which contains the conserved active site triad of Cys272, His330, and Asp353 residues (using TGase 3 residue numbers); a β-barrel 1 domain; and a β-barrel 2 domain at the carboxy terminus (Fig. 1). In the X-ray structures of TGase 3, the catalytic triad active site residues,

Table 1
Summary of data on roles of metal ions in TGase 3

	Form	Site 1	Site 2	Site 3	Channel	TGase activity	Resolution (Å)	PDB (ID)
I	Zymogen	Ca^{2+}			Closed	No	2.20	1L9M
II	Zymogen proteolyzed with dispase	Ca^{2+}	Ca^{2+}	Ca^{2+}	Open	Yes	2.70	1NUD
III	II + purified on MonoQ column	Ca^{2+}			Closed	No	2.70	1NUF
IV	III + $CaCl_2$	Ca^{2+}	Ca^{2+}	Ca^{2+}	Open	Yes	2.10	1L9N
V	III + $CaCl_2$ + $MgCl_2$ + ATP	Ca^{2+}	Ca^{2+}	Mg^{2+}	Closed	No	2.40	1NUG
VI	III + $CaCl_2$ + $MgCl_2$ + GTPγS	Ca^{2+}	Ca^{2+}	Mg^{2+}	Closed	No	2.10	1RLE
VII	III + $CaCl_2$ + $MgCl_2$ + GDP	Ca^{2+}	Ca^{2+}	Ca^{2+}/Mg^{2+}	Open/Closed	Yes/no	1.90	1VJJ
VIII	III + $CaCl_2$ + $MgCl_2$ + GMP	Ca^{2+}	Ca^{2+}	Mg^{2+}	Closed	No	2.00	1SGX

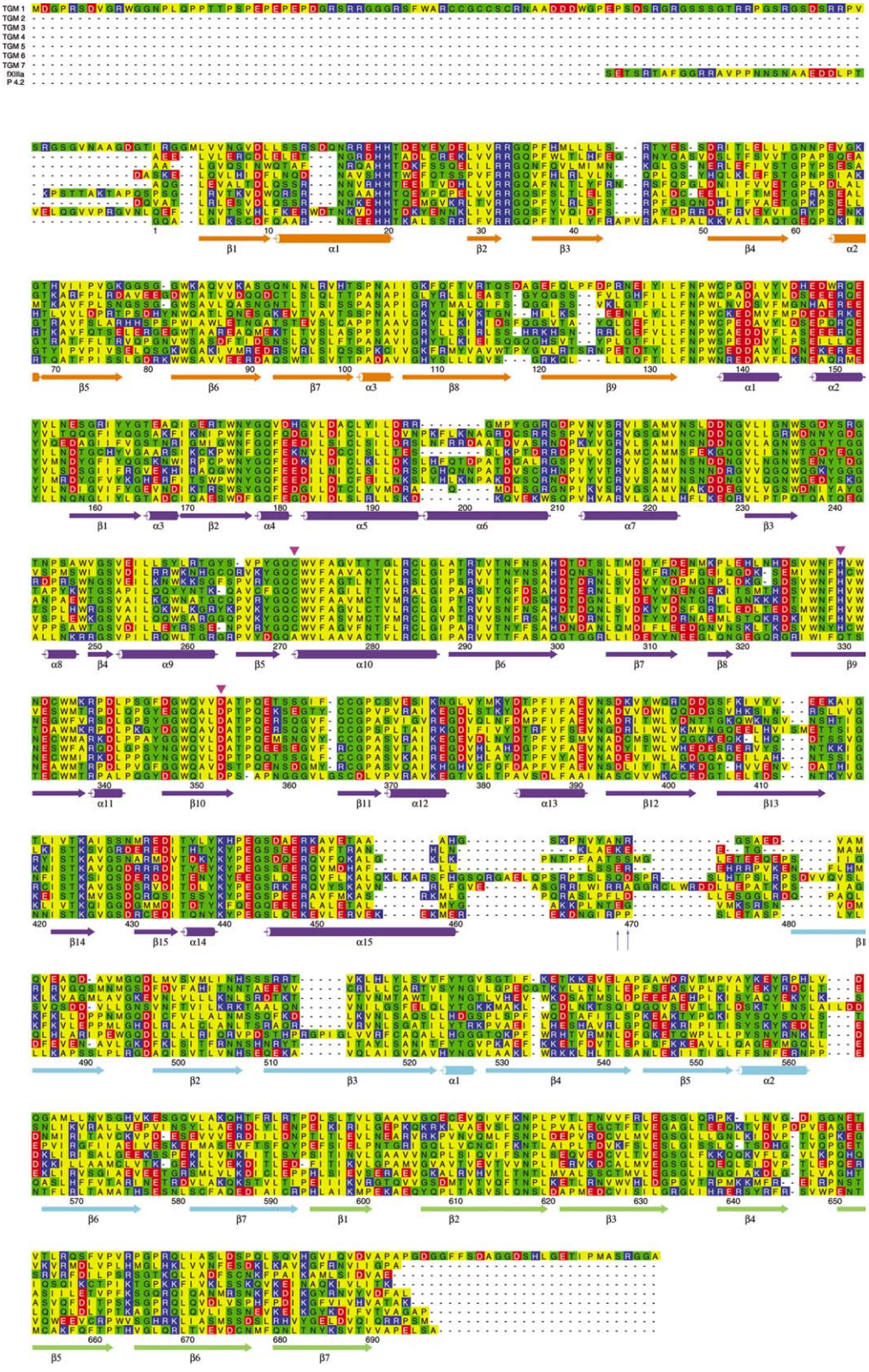

Fig. 1. The amino acid sequence alignment of nine TGase enzymes of the human genome. The catalytic triad Cys272, His330, and Asp353 are indicated by a triangle in *magenta*. The cleavage site is shown with double arrow (*blue*) which is located on a long flexible loop that connects the catalytic core domain and the β-barrel 1 domain. The amino acids highlighted in *red* are acidic (D,E), *blue* basic (H,K,R), *yellow* nonpolar (A,I,L,V,P,F,W,M), and *green* polar (G,S,T,Y,C,N,Q), respectively. The sequence numbering and secondary structure assignment is based on TGase 3. The secondary structure domains are the β-sandwich (*orange*), the catalytic core (*purple*), the barrel 1 (*cyana*), and barrel 2 (*green*).

including the Cys272 residue at the active site, are buried in the hydrophobic interior of the enzymes (Fig. 2A). The sulfhydryl group of the Cys272 forms an intimate thiolate–imidazolium ion pair with His330. The imino nitrogen atom of the His330 ring forms a hydrogen bond with the terminal oxygen atom of Asp353. In addition, the hydroxyl of Tyr525 is within hydrogen bonding distance of Cys272 and Trp236 and is located at loop of the sequence motif Ile523-Asn526 of the β-barrel domain 1 that occludes the entrance to the active site. These bonds must be cleaved and structural motifs near the enzyme's surface must be moved to allow substrates to approach the active site to effect reaction.

4. Metal binding sites

There has been some difficulty in purifying the TGase 3 zymogen to molecular homogeneity from certain as yet uncharacterized low molecular weight calcium binding proteins, which implies that the enzyme may coexist in vivo in the cytosol with either its natural protease(s) and/or sources of Ca^{2+} ions required for transamidation (Folk and Chung, 1985; Lorand and Conrad, 1984; Melino et al., 2000). By use of biochemical assays, it has been shown that the TGase 3 zymogen constitutively binds one Ca^{2+} ion very tightly. Upon proteolysis, two additional Ca^{2+} ions readily bind, and the enzyme becomes fully active (Ahvazi et al., 2002a,b).

The crystal structures have now shown clearly that three Ca^{2+} ions are located in the catalytic core domain of TGase 3 (Fig. 2A). The ion in site one is also seen in the equivalent position within the zymogen, this site being located 13.7 Å above the active site Cys272 residue. In the activated enzyme, a small loop Val231-Asn235 moves over somewhat to effectively bury this Ca^{2+} ion in the interior of the enzyme, and the ion changes its coordination from six to seven, more typical for Ca^{2+} ions. The Ca^{2+} ion in site two is located about 23 Å below Cys272. Its location is similar to that previously reported for fXIIIa (Fox et al., 1999). The Ca^{2+} ion in site three is located about 22 Å from Cys272. Notably, binding in site three moves a loop on the 'front' surface of the enzyme by about 9 Å so that residue Asp324 can coordinate with the Ca^{2+} ion. This loop movement opens a channel through the enzyme (Fig. 2B). Moreover, the opening of the channel exposes side chains of Trp236 and Trp327 on the upper and outer surface of the channel that are known to participate in the reaction mechanism (Ahvazi and Steinert, 2003; Iismaa et al., 2003; Murthy et al., 2002).

There are additional structures of TGase 3 in the presence of Ca^{2+} and/or Mg^{2+}which provide further insights on the contribution of each Ca^{2+} ion to activation and activity (Ahvazi et al., 2003). Together, the data above lead to the emerging picture that Ca^{2+} ion manipulates the channel opening required for substrate access, through calcium sites two and three. Should one site acquire a Mg^{2+} ion, the enzyme will become inactive by closing the channel opening to the active site (Ahvazi et al., 2003). Identification of metal ions in each structure was based on a number of criteria. The coordination number of six with an octahedral geometry is more consistent with Mg^{2+} ligation. In contrast, Ca^{2+} ion favors seven ligands with a pentagonal bipyramid heptacoordination. When a water is modeled into the density, there was an unusually low $\langle B \rangle$ factor value associated for water relative to the surrounding sidechains atoms. But when an appropriate Mg^{2+} was assigned to the density, the $\langle B \rangle$ value was similar to those of the surrounding residues. The short distances to nearby oxygen atoms (2.0–2.1 Å), also support the identification of the Mg^{2+} ion. The bond distances to the metal–oxygen ligand atom typically range from 2.0 to 2.1 Å for Mg^{2+} and 2.1–2.8 Å for Ca^{2+}, respectively. Moreover, the bond-valence calculation parameters result in further support of an assignment of the metal site. Finally, an inductively coupled plasma-mass spectrometry dynamic reaction cell based analysis of crystals indicated the existence of correct metals.

It is conceivable that the channel opening could be manipulated and controlled solely by intracellular cation levels. Calcium is an important physiological regulator of epithelial differentiation in vivo and in vitro. The main evidence that calcium plays a role in vivo is the presence of a Ca^{2+} gradient in epidermis that increases from the basal to the granular cell layer (Menon and Elias, 1991; Menon et al., 1985). In vitro, epidermal keratinocytes grown in low calcium concentrations continue to proliferate and lack desmosomes, but when shifted to media containing higher calcium concentrations greater than 0.1 mM they express differentiation markers such as involucrin, K1, K10, loricrin, and profilaggrin. High calcium concentrations are necessary for stratification and desmosome assembly (Yuspa et al., 1989; Menon et al., 1994). The physiological concentration of free Mg^{2+} ions in resting eukaryotic cells is 2 mM, whereas that for free Ca^{2+} ions is approximately 100 nM, so that there is about a 10^4 molar ratio (Niki et al., 1996).

5. Substrate access to the active site

An early suggestion on a possible TGase enzyme mechanism proposed that substrates should approach from the front side of the enzyme, dislodge several areas on the surface to expose the two Trp residues (implicated to be important through mutagenesis studies) and to break the hydrogen bonds masking the active site Cys residue (Noguchi et al., 2001; Yee et al., 1994). The crystallographic data on the structures of the active forms of TGase 3 reveal that the side chains of these Trp

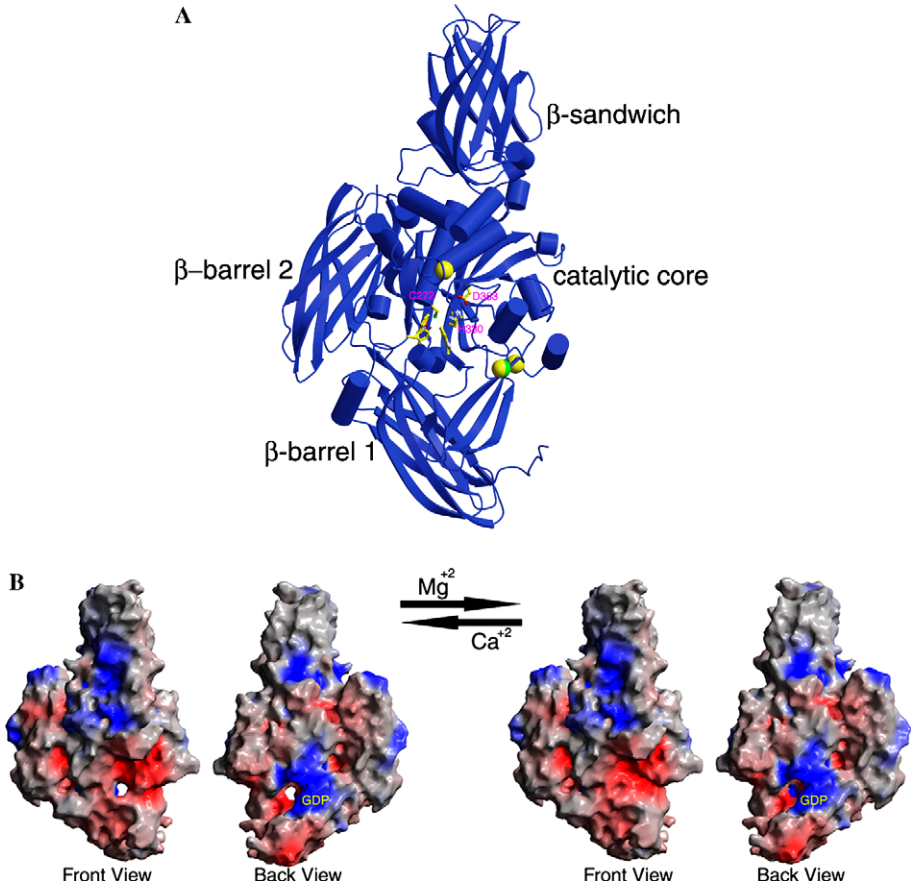

Fig. 2. (A) The structures of the activated human TGase 3 enzyme form in presence of Ca^{2+}. The amino-terminal β-sandwich, catalytic core, β-barrel 1, and β-barrel 2 domains are shown. The Ca^{2+} ions are shown in *yellow*, the sole Mg^{2+} ion in *green*. The active site residues Cys272, His330, and Asp353 are buried and inaccessible. The Tyr525, Trp236, and Trp327 residues are also shown. (B) Below are shown the electrostatic potential surfaces. The acidic and basic residues are *red* and *blue*, respectively. The electrostatic potential scale is from −15 kT (*deep red*) to +15 kT (*deep blue*). The open channel is clearly evident upon Ca^{2+} binding in site 3 however, the 'front' side remains closed upon exchange of Ca^{2+} by Mg^{2+} ion.

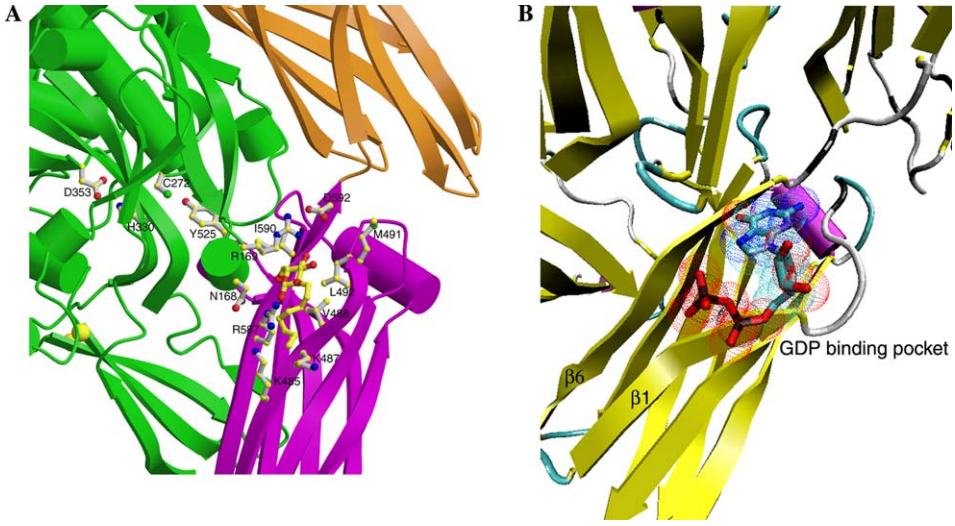

Fig. 3. (A) The secondary structure associated with the 'back' of activated TGase 3 enzyme. The location of β-octylglucoside molecule is shown as ball and stick and is located in the same binding pocket as TGase2–GDP complex. (B) The location of GDP molecule in activated human TGase 3 is shown. A view of the GDP nucleotide-binding site of TGase 3 consists of residues Asn168 and Arg169 from the core domain and the residues Lys485 and Lys487 from the first β-strand of the β-barrel 1 and Arg588, Asp589, Ile590, and Asp592 from the last β-strand of the β-barrel 1.

residues are indeed exposed after binding of Ca^{2+} ions in sites two and three, and thus control at least one key step in 'opening' of the enzyme for reaction (Ahvazi et al., 2002a,b, 2003). We are intrigued by the observed channel modulation directed toward the active site in TGase 3, as this channel appearance coincides closely with the activation of the enzyme. It appears likely that the two sides of this channel could serve as 'ports of entry' for the two substrates. However, the channel is about 16 Å deep from the bulk solvent at the backside to the active site Cys272 residue and only 13 Å wide at its widest point. Also, the side chains of a number of residues, in particular the guanidinium groups of Arg396, Arg420, and Arg570, protrude into the channel's cavity. Moreover, these insights impose some constraints on how this cavity/channel could be used by substrates without further structural rearrangements: the channel observed in the absence of substrate is too deep for a Gln or Lys substrate residue side chain to penetrate to the active site Cys residue; and it is too narrow for a peptide chain backbone bearing a reactive Gln or Lys residue to penetrate through the maze of Arg side chains to the active site Cys residue (Ahvazi and Steinert, 2003).

6. Guanine nucleotide binding

Among the TGase enzymes, only TGase 2 has been shown to bind to GTP (Nakaoka et al., 1994). The corresponding GDP binding site in TGase 2 was revealed recently (Liu et al., 2002). In the activated TGase 3 form, a β-octylglucoside detergent molecule that is used in the crystallization condition appeared to be occupying the equivalent hydrophobic pocket (Fig. 3A). We conducted new crystallographic studies on the complex of TGase 3 with both GTPγS and GDP, providing clear evidence that this enzyme also uses this pocket for binding to guanine nucleotides (Ahvazi et al., 2004). In addition, using an extensive set of biochemical and crystallographic data we could further uncover how TGase 3 enzyme interacts with GTP and in a coordinated manner with Ca^{2+}/Mg^{2+}. Clearly, TGase 3 is able to hydrolyze GTP to GDP effectively (Ahvazi et al., 2004). All of this was surprising, as the amino-acid side-chains that lie in the GDP binding pocket of TGase 2 are not precisely conserved in TGase 3 (Fig. 3B). The crystallographic studies on TGase 3–GTPγS/GDP complexes have therefore challenged the long-standing notion that among all TGases, only TGase 2 binds GTP, and hydrolyzes it to GDP (Iismaa et al., 2000; Im et al., 1990; Lee et al., 1989) as shown in Fig. 4.

Interestingly, the GDP binding pockets of TGase 3 and TGase 2 are related to each other in their position and in several key amino acid determinants, but these pockets are only superficially related to those characterized in G-proteins. The biochemical studies are now clearly testing and supporting the crystallographic inferences. GTP binding by TGase 3 inhibits the enzyme's transamidation activity, whereas calcium binding inhibits GTP binding and activates the enzyme (Ahvazi et al., 2004). These results further suggest that the binding of GTP and divalent cations might act as a coordinated molecular switch to regulate the activity of TGase 3–GTP binding and is coordinated with a substitution of one of the three Ca^{2+} binding sites, and other subtle changes that cause the closing of a central channel leading to the enzyme's active site. Hydrolysis of GTP to GDP appears to impart a two-state conformation representing both the GTP-bound enzyme (the GTPγS inactive state with Mg^{2+} ion at site 3/closed channel) and the non-nucleotide bound (the active state with Ca^{2+} ion at site 3/open channel) of the enzyme. A structure based sequence homology among the TGase

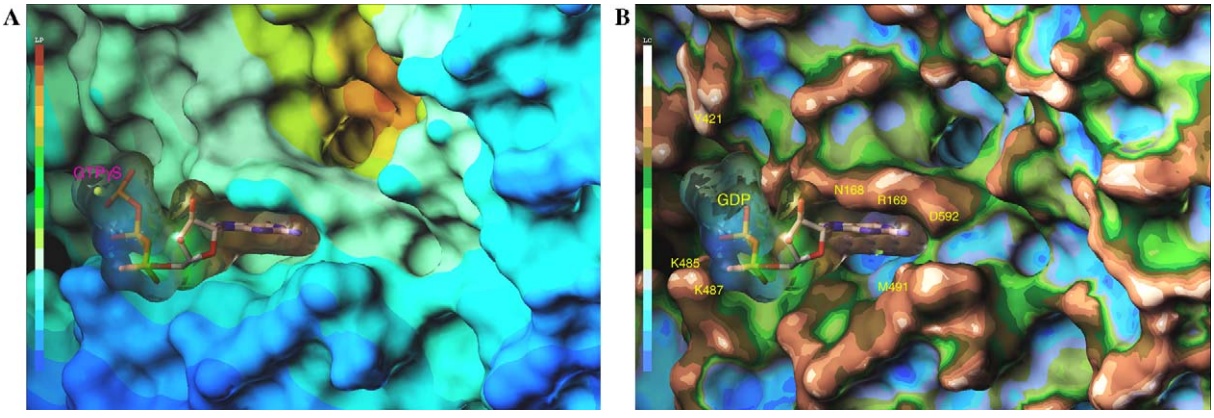

Fig. 4. (A) The lipophilic (brown) and hydrophilic (blue) surface properties of GTPγS binding pocket. (B) The color ramp for local curvature surface of GDP ranges from white (highly convex, positive curvatures) to blue (highly concave, negative curvature). The surrounding residues within 6.5 Å of the GDP nucleotide located at the interface of the core domain (top) and the β-barrel 1 domain (bottom). The GTPγS and GDP are shown in ball-and-stick.

enzymes goes even further to suggest that besides TGase 2 and TGase 3, many other members of the TGase family would be able to bind to GTP/GDP (Fig. 1).

7. Future directions

It is known that GTP binding by TGase 3 inhibits calcium binding and thus the TGase transamidation activity, whereas calcium binding inhibits GTP binding and promotes the enzyme activity (Ahvazi et al., 2004). Thus, a remaining question to be resolved is to how guanine nucleotide and calcium ions affect each other within the structure of these enzymes in a manner that has direct consequences for the transamidation activity. This has not been well addressed, in part because the TGase3–GDP complex structure did not show any substrate bound that normally associates with the enzyme. As such, it is not clear yet whether calcium ions and the guanine nucleotide share the same amino acid contacts or exert opposite influences with respect to the active site through a bridging structural element. Also, lacking is information as to how substrates positioned at the active site could influence the binding or hydrolysis of the GTP. These issues should be resolved with the next generation of structural studies that examine nucleotide- and substrate-complexes of TGase 3.

8. Figures

The figures in this report were prepared with Molscript (Kraulis, 1991) Raster3D (Merritt and Bacon, 1997), and GRASP (Nicholls et al., 1991) programs.

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