MTG: a versatile tool for site specific conjugation

by Ralf Pasternack, Katrin Bott-Fischer and Martin Hils (2020)

Transglutaminases are defined as R-glutaminyl-peptide: amine γ-glutamyl-transferase (EC 2.3.2.13). They use a modified double-displacement mechanism to carry out an acyl transfer reaction between the γ-carboxamide group of a peptide-bound glutamine residue and the ε-amino group of a peptide-bound lysine (Figure 1). The active site in mammalian transglutaminases consists of a catalytic triad (Cys, His and Asp). The active site cysteine reacts with the γ-carboxamide of the glutamine, forming a γ-glutamyl thioester resulting in the release of ammonia. This activated species subsequently reacts with nucleophilic primary amines, yielding either an isopeptide bond (pathway ①) or a (γ-glutamyl)amide bond (pathway ②). When an amine is not available, the acyl-enzyme intermediate can be hydrolyzed to yield glutamic acid (pathway ③).

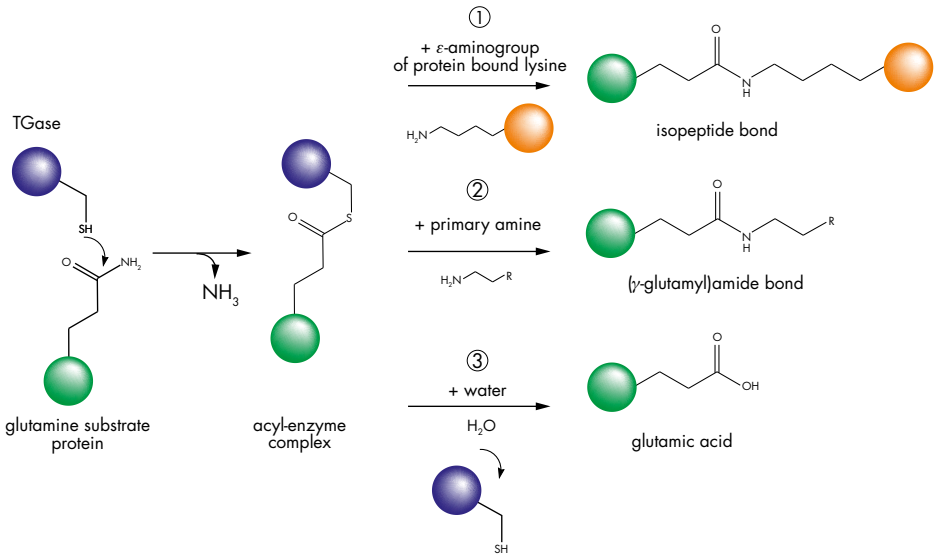

Figure 1: Reaction pathways of transglutaminase.

Today, we know that transglutaminases are produced by species of all taxonomic domains. Transglutaminases were described from microorganisms, plants, invertebrates, amphibians, fish, birds and mammals (for review see Gerrard et al. 2001, Griffin et al. 2002). In humans, even nine transglutaminase genes are present, one coding for an inactive membrane protein, eight for active transglutaminases (Griffin et al. 2002). Important to mention, eukaryotic transglutaminases are not related to microbial transglutaminases, but the result of convergent evolution. Essentially, eukaryotic transglutaminases require Ca^{2+} ions as cofactor, while microbial transglutaminases catalyzes the cross-linking reaction without cofactor requirement.

Microbial transglutaminase (MTG or synonymous BTG for bacterial transglutaminase) is a versatile tool for conjugation of proteins or peptides with small molecules, polymers, surfaces, DNA or other proteins (Strop 2014). It yields site specifically labeled, homogenous products. Many applications have been described in scientific literature, including pegylation, labeling with fluorescent dyes and conjugation of enzymes (Kamiya et al. 2009; Pasternack et al. 1998; Sato 2002; Maullu et al. 2009; da Silva Freitas et al. 2013). However, today the manufacturing of antibody drug conjugates (ADCs) is the main application field for MTG.

In *Streptomyces mobaraensis*, the MTG-gene is translated into a prepro-enzyme consisting of 407 amino acid residues. The hydrophobic 31 amino acid long pre-peptide is required for efficient protein secretion. It is followed by the 45 amino acid pro-peptide which renders the enzyme inactive (zymogen). This protects the strain from uncontrolled cross-linking reactions. The extracellular pro-enzyme is activated by cleavage of the N-terminal pro-peptide (~ 5 kDa). The mature enzyme has a mass of 38 kDa (Pasternack et al. 1998; Washizu et al. 1994) and an isoelectric point at pH 8.9 (Ando et al. 1989). The primary structure is given in Figure 2.

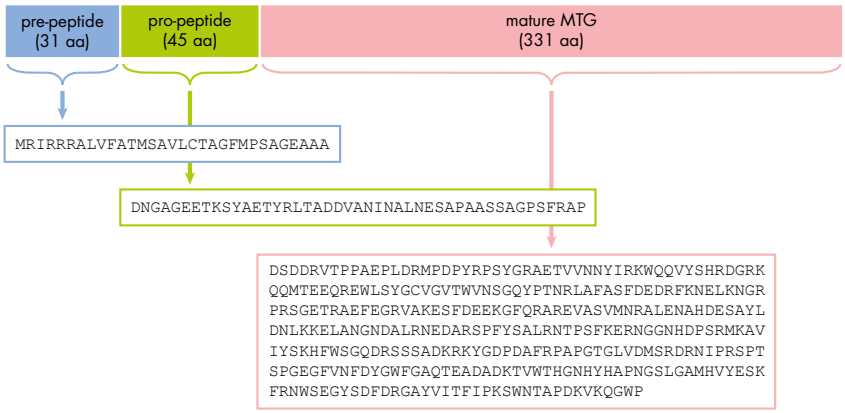

Figure 2: Primary structure of microbial transglutaminase prepro-enzyme. Figure adapted from Kanaji et al. 1993; Kashiwagi et al. 2002; Pasternack et al. 1998.

Antibody drug conjugate formation by MTG

An emerging field in oncology and beyond are antibody drug conjugates (ADCs). In ADC development, the aspects of antibody, payload and linker technology need to be taken into consideration (Figure 3). Each part of the ADC needs to meet high demands in order to provide high efficacy already by low drug doses, which in combination reduces the potential for adverse reactions.

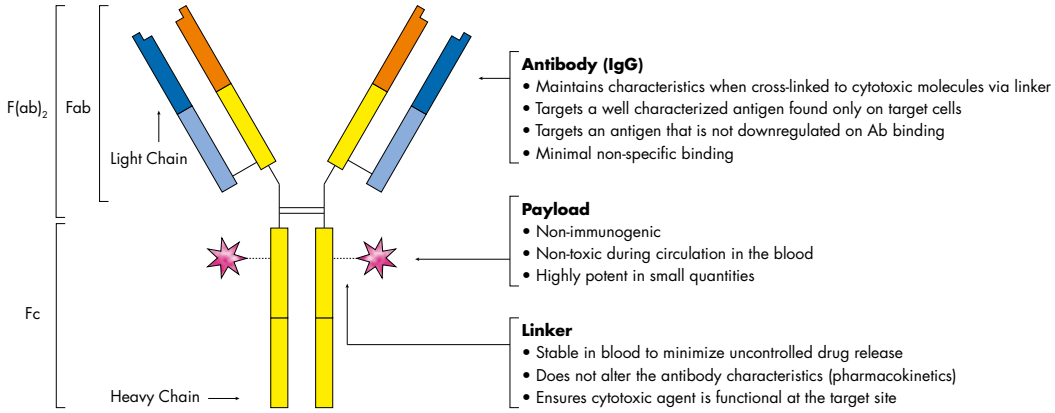

Figure 3: Schematic structure of IgG antibody drug conjugates. Figure adapted from Zolot et al. 2013 and Mulisch 2014.

Resulting from the antibody's high selectivity, the minimal effective dose can be delivered to a target (e.g. cancer cell) leading to low unspecific binding though providing the maximal tolerated dose (Schumacher et al. 2016).
The mode of action of ADCs is based on their degradation in cell's lysosomes and subsequent payload release. To achieve that, the antibody first needs to bind the antigen at the tumor cells surface. Receptor mediated endocytosis in early endosomes imports the ADC into the cell. If the ADC's Fc-domain binds to the FcRn-receptor of the endosome, it is re-exported out of the cell. Thus, antibodies to be used as ADC should exhibit low or no binding to FcRn.
Late endosomes finally fuse with lysosomes leading to proteolytic degradation of the ADC. Depending on the selected linker technology, the payload is released either by low pH or by proteolysis. The cytotoxic payload, usually DNA-binding or microtubule polymerization inhibiting substances, leaves the lysosome and can access DNA or microtubules, leading to immediate cell death or apoptosis initiation (Peters and Brown 2015).
Drawback of this ADC mode of action can be the so called "Bystander Effect", where the released drug diffuses through the cell membrane and affects neighboring healthy cells (Bouchard et al. 2014).

ADCs can be generated using chemical, physical or enzymatic conjugation. For all methods mild reaction conditions are required, in order to maintain the antibody's native structure and functionality. Homogenous conjugates guarantee batch independent drug efficacy, required for pre-clinical and clinical development and assessment of adverse effects (Kline et al. 2015).

MTG can label native antibodies, especially IgGs and can therefore be used for the generation of ADCs.

IgGs heavy chains from various species exhibit a conserved sequence of Q295[F/Y]N. Q295 is recognized by MTG as substrate. However, the Asp297 in proximity is glycosylated leading to sterical hindrance of MTG binding and catalysis (Figure 4). Deglycosylation of Asp297 by the enzyme PNGase F, rendering Gln295 accessible for microbial transglutaminase, resulting in 2 labels pers antibody (1 per heavy chain, Jeger et al. 2010; Dennler et al. 2014). MTG mediated antibody conjugation is depicted in Figure 5.

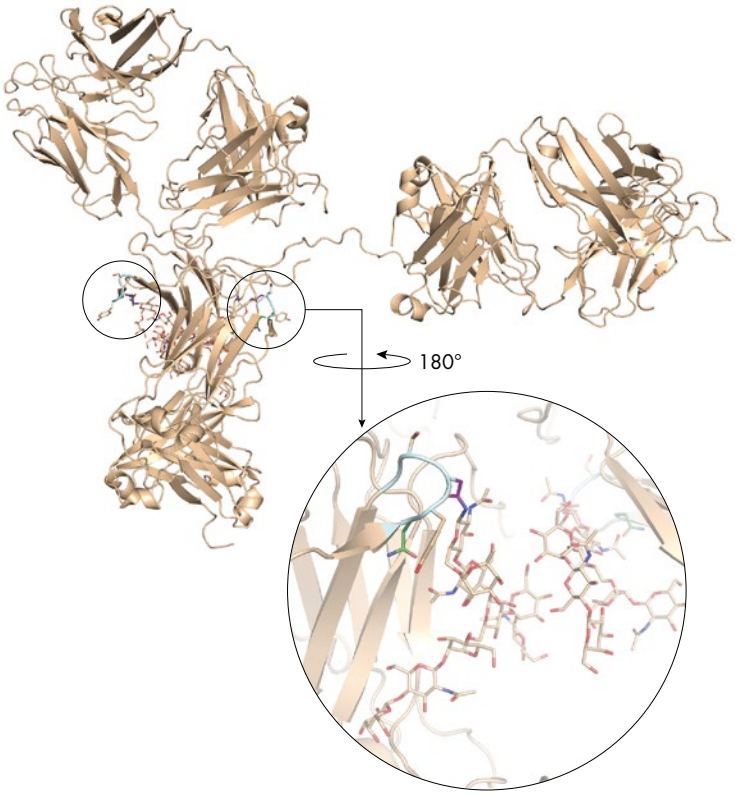

Figure 4: Crystal structure of human IgG. Enlarged: Gln295 and glycosylated Asp297 (black circle). Green: Glutamin 295; Purple: Asparagine 297; Blue: backbone of C/E-Loop QYNST. Crsytal structure from Saphire et al., 2001 (PDB-ID: 1HZH, Bachelor thesis of S. Bitsch, Zedira, 2016).

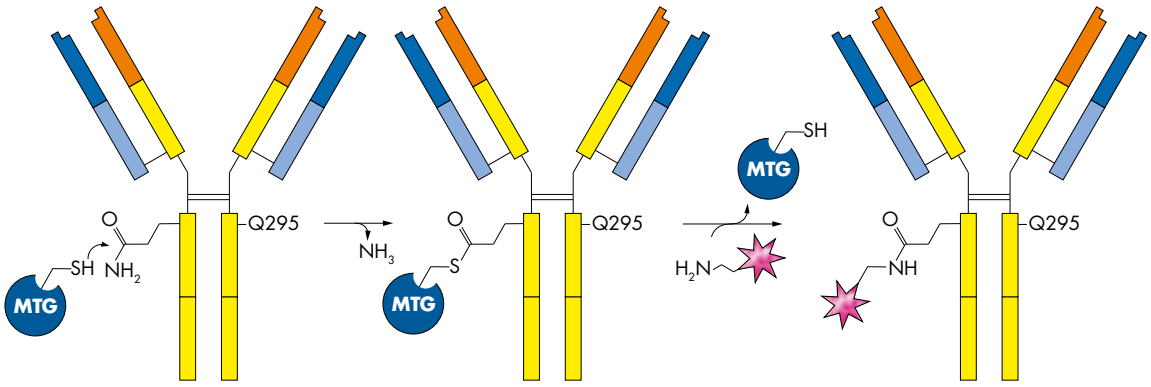

Figure 5: MTG reaction pathway. Antibody heavy chains are conjugated with a drug (linked to a primary amine) by MTG on position Q295, resulting in an ADC with two site specifically conjugated drug molecules. Here, the conjugation of only one Q295 is shown.

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An alternative to deglycosylation was discovered by Spycher et al. Using lysine-containing peptides as primary amine substrates results in efficient labeling without deglycosylation (Spycher et al. 2019). The technology is now available at the swiss company Araris Biotech AG.
The Rinat-Pfizer group engineered the transglutaminase recognition sequence (Q-tag) LLQA to several positions of the heavy and light chain of IgG and successfully conjugated fluorophores and auristatin derivates resulting in drug to antibody ratio (DAR) 1.2 - 2 (Strop et al. 2013). However, the MTG mediated conjugation lead to unspecific reactivity at Q295, which could be avoided by Q295N mutation of IgG's heavy chain (Farias et al. 2014).
The Darmstadt, Germany, based academic groups of Harald Kolmar and Hans-Lothar Fuchsbauer commonly designed Q-tag sequences based on microbial transglutaminase's natural substrates DAIP and SPI_P (Siegmund et al. 2015; Ebenig 2019), showing improved reaction kinetics. Here, to avoid intermolecular cross-linking of IgG the terminal K447 had to be removed.
A further Q-tag sequence named CovIsoLink™ was developed by the French Company Covalab (El Alaoui and Thomas 2016). They showed that sequence environment, conformation of the antibody, and type of spacer can influence the conjugation. Conjugation was improved when the Q-tag was fused to the heavy chain's C-terminus in comparison to the light chain's C-terminus (Martin et al. 2018).
An alternative to conjugation at glutamine residues was developed by Morphotek/Eisei (Spidel and Albone 2019; Spidel et al. 2017) using antibody's lysine-residues and glutamin containing peptides as substrates. In analogy to the Q-labeling approach, either native or engineered lysines can be addressed for conjugation.

Your conjugation tool is availabel at Zedira

Zedira provides MTG-products suitable for development purposes as well as for the production of ADCs for clinical studies. MTG related quality features (purity, specific activity) as well as impurity thresholds (host cell protein and host cell DNA content, endotoxin content, and sterility) are met.
MTG can be stored at -80°C for at least 3 years. Upon reconstitution it is stable for at least 10 freeze-thaw cycles.

Art. No.	Name	Unit	Price
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	(MTG, recombinantly produced in ***E. coli***,	**10 x 25 U**	**2950 €**
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