Screening for the Preferred Substrate Sequence of Transglutaminase Using a Phage-displayed Peptide Library

**IDENTIFICATION OF PEPTIDE SUBSTRATES FOR TGASE 2 AND FACTOR XIIIA**

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Mammalian transglutaminase (TGase, 2.3.2.13) is an enzyme that catalyzes the formation of isopeptide cross-links between glutamine and lysine residues in a variety of proteins and also attaches other primary amines to peptide-bound glutamines (1–5). To date, eight human TGase isozymes (Factor XIII, TGases 1–7) have been identified, comprising a large protein family with unique tissue distributions and physiological roles. Among the isozymes, TGase 2 and Factor XIII are two major members, although their locations and regulation differ. TGase 2 is expressed ubiquitously and has been implicated in many biological processes, including apoptosis, stabilization of the extracellular matrix, and regulation of growth factors (6, 7). Plasma Factor XIII is synthesized as azymogen that comprises two A and two B subunits (8). The A subunit contains the catalytic domain and is converted by thrombin-dependent proteolysis into its active form during clot formation. Activated Factor XIII (Factor XIIIa) is involved in fibrin stabilization and wound healing (9).

Transglutaminase (TGase2, 2.3.2.13) is an enzyme that catalyzes the formation of isopeptide cross-links between glutamine and lysine residues in a variety of proteins and also attaches other primary amines to peptide-bound glutamines (1–5). To date, eight human TGase isozymes (Factor XIII, TGases 1–7) have been identified, comprising a large protein family with unique tissue distributions and physiological roles. Among the isozymes, TGase 2 and Factor XIII are two major members, although their locations and regulation differ. TGase 2 is expressed ubiquitously and has been implicated in many biological processes, including apoptosis, stabilization of the extracellular matrix, and regulation of growth factors (6, 7). Plasma Factor XIII is synthesized as azymogen that comprises two A and two B subunits (8). The A subunit contains the catalytic domain and is converted by thrombin-dependent proteolysis into its active form during clot formation. Activated Factor XIII (Factor XIIIa) is involved in fibrin stabilization and wound healing (9).

Generally, TGase reactions involve a Ca2+-dependent acyl transfer via a double displacement mechanism. In the first step, a glutamine γ-carboxyamide group in the substrate binds to a cysteine at the active site, resulting in formation of a γ-glutamylthioester bond and release of ammonia. Formation of the covalent acyl enzyme intermediate is the rate-limiting step. The e-amino group of a peptide-bound lysine, as a nucleophilic substrate, binds to the acyl enzyme intermediate and then attacks the thioester bond, thereby generating an intermolecular isopeptide e-(γ-glutamyl)lysine cross-link. Primary amines can replace lysine in transamination reactions and become incorporated into the substrate proteins. Additionally, water can act as a nucleophile, resulting in deamidation of the glutamine residue and formation of a glutamic acid residue.

However, the mechanisms by which TGases recognize substrates remain poorly understood. During the first step, TGases are selective with regard to which glutamine residues in the substrate protein take part in the reaction; they are much less selective with regard to the lysine residue or primary amines. Although partial amino acid sequences have been identified from a variety of highly reactive substrates (10–17), fewer consensus sequences and structures have been identified around the reactive glutamine residues (5, 18). Investigations using the TGase substrate protein data base have produced information regarding TGase substrate sequence preferences (19, 20). Additionally, in studies on celiac disease, in which an unusual gluten-derived peptide antigen is produced, the amino acid distribution around the glutamine residue has been investigated to determine the deamidation sites preferred by TGase 2 (21–24). Although several preferred substrate sequences have been suggested by these studies, no research has been performed to date using an unbiased peptide library for identification of favorable primary structures.

To study the amino acid sequences around reactive glutamine residue(s) that are preferred by TGases, we used an unbiased phage display random peptide library. In this system, peptides are expressed as fusions with the M13 bacteriophage coat protein pIII, displaying the random peptide sequence on the phage surface (25, 26). This system has been used for production of antibody-like molecules, epitope identification, and characterization of substrate recognition sites for various proteases (27–29).

We screened a phage-displayed random peptide library using avidin affinity purification of phage clones that incorporated a biotin-labeled primary amine in catalytic reactions with TGase 2 or Factor XIIIa. In the peptide sequences displayed on selected phage clones, we observed a notable tendency toward a preferred primary sequence around the reactive glutamine. These identified peptide sequences were assessed as recombinant fusion proteins with a modified GST (glutathione S-transferase). Selected peptides demonstrated a significant isozyme-specific incorporation of primary amine and blocking effects on cross-linking...
reaction. This report describes the establishment of a screening system that is equally applicable to the search for substrate sequence preferences in other TGases.

**EXPERIMENTAL PROCEDURES**

**Transglutaminases**—Guinea pig liver TGase 2 was kindly provided by Dr. Ikura (30). Human factor XIII (Fibrogammin®P; ZLB Behring, Marburg, Germany) was activated by treatment with bovine thrombin (Sigma). Prior to use for phage display screening, thrombin was inactivated by addition of phenylmethylsulfonyl fluoride. The enzymatic activities were confirmed using microtiter plate assay, which measures the incorporated amounts of biotinylated primary amine into dimethyl-casine (DMC) (Sigma), as described later.

**Screening Using Phage Display Random Peptide Library**—An M13 Ph.D.-12 phage display system (New England Biolabs Inc.) was used for screening of preferred substrate sequences in both the TGase 2 and Factor XIIIa catalytic reactions. Approximately $1.5 \times 10^{11}$ (1st-round panning) or $1 \times 10^{12}$–$13$ (2nd-5th-round panning) phage clones were incubated at 37 °C with TGases in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl (TBS buffer) containing 1 mM DTT, 5 mM CaCl$_2$ and 5 mM biotinylated cadaverine (Bio-Cd) (EZ-link™ 5-(biotinamido)pentylamine) (Pierce). To be included as a similar enzymatic activity in the reaction mixture, TGase 2 and Factor XIIIa were added at final concentrations of 0.5 and 5 ng/µl, respectively. In each screening round, TGase reaction was performed for 15, 10, and 2 min in reactions for 1st, 2nd, and 3rd–5th rounds, respectively. The reaction was terminated by the addition of 20 mM EDTA. Phage particles were then precipitated by addition of polyethylene glycol and NaCl to remove unbound Bio-Cd in the presence of salmon sperm DNA as a carrier. Next, phage clones that covalently incorporated Bio-Cd were selected by affinity chromatography using mono-avidin gel (SoftLink™ Soft Release Avidin Resin; Pro-mega Corp.). After washing with TBS containing 0.1% Tween 20 and 2 mM EDTA four times and then with TBS twice, the bound phage clones were eluted by TBS containing 5 mM biotin. The entire eluate was used to infect early log phase ER2738 host bacteria, and the phages were amplified by culture for 4.5 h at 37 °C. The phage DNA fragment encoding the displayed 12-mer peptide (SVTVQQEL, the target sequence from bovine crystalline (17), was constructed by PCR-based site-directed mutagenesis according to the method. The phage DNA fragment encoding the target peptide) were annealed and inserted into the NcoI and EagI site of pET24d-GST(QN). The resulting plasmid was able to express the phage-displayed peptide at the N terminus. In recombinant proteins, the additional peptide sequence for MDSHS was attached at the N terminus of each peptide.

The GST(QN) fusion proteins with several peptide sequences from previous reports were also expressed and purified, as described later. For example, the expression vector for GST(QN) fusion protein with TVQHEL, the target sequence from bovine crystalline (17), was constructed using pET24d-GST(QN). Oligonucleotides encoding amino acid sequence (forward, 5′-CATGGATCCACTCTAGCTTCAAGAATTAGGTTGAGGTTTC-3′; reverse, 5′-GGCCGACACCCTAACCTTTCTGTTGTT (QQIV) and CCTGGTGTCACAAAGATTGTT (PGGQIV); trappin (13) GCTGCAAATGCTTATATAA (QQQVSPLTGLK); substance P (15), CGTCCTAAACCTCAACAATTTTGTGGTTTATAG (RPKQPOFF-GLM); β-endorphin (22), ACTTCTGAAATCTCAACCTCTTTAGTTACT (TSEKSSQPTLVT).

**Expression and Purification of GST Fusion Proteins**—Escherichia coli BL21(DE3)LysS was transformed with each constructed expression plasmid for GST(QN) fusion protein. Production in bacteria was induced with 1.0 mM isopropyl β-D-thiogalactoside, and the recombinant proteins were purified from soluble fraction of the extract using the manufacturer’s method with TALON metal affinity resin (BD Bioscience). The protein concentration of the purified protein was determined by the Bradford method (Bio-Rad Laboratories).

**Assessments of Substrate Preference in Transglutaminase Reaction**—TGase reactivities of recombinant GST(QN) fusion proteins were evaluated by incorporation of monodansylcadaverine (Dansyl-Cd) (Sigma), a fluorometric-labeled cadaverine. The assay mixture was TBS buffer containing 5 mM CaCl$_2$, 1 mM DTT, 200 ng/µl of recombinant protein, and 0.5 mM Dansyl-Cd in the presence of either TGase 2 (0.5 ng/µl) or Factor XIIIa (5 ng/µl). As a standard, 200 ng/µl of DMC was used for each assessment. The reaction mixture was incubated at 37 °C and then separated on 12.5% SDS-PAGE. A fluorograph of the gel was obtained on a UV lightbox (254 nm) to detect the Dansyl-Cd incorporation. Fluorescence intensity was measured by scanning the photograph of the gel and analyzing with the imaging software SCION (Scion Corp., Frederick, MD).

In the case of incorporation assay using radiolabeled primary amine, $[^{3}H]$putrescine (PerkinElmer Inc.) was used at a final concentration of 1.0 mM in the reaction mixture. Recombinant protein (1.6 µg/µl) was incubated with TGase 2 (4 ng/µl) or Factor XIIIa (40 ng/µl) in TBS buffer containing 5 mM CaCl$_2$ and 1 mM DTT. Reaction products were fixed on trichloroacetic acid-containing filters, which were washed with 10% trichloroacetic acid solution twice and then dried. Residual radioactivity on the filters was measured using a liquid scintillation counter.

**Substrate Assessment using Synthetic Peptides**—Two representative 12-mer peptides ( pepT26: H9QSYDPWMDH) and ( pepF11K: DMQMLLPWPAVAL) were purchased from Peptidesupport Inc. (Kitakyushu, Japan) in biotinylated form and confirmed by high-per-
formance liquid chromatography and time-of-flight mass spectrometry. The peptides were dissolved in dimethyl sulfoxide (Me2SO) at the final concentration of 100 mM as a stock solution.

To evaluate substrate reactivity and specificity as synthetic peptides, microtiter plate assay was performed with some modification (31, 32). Briefly, 200 ng/ml of bovine casein (Nacalai Tesque Inc.) was incubated with each peptide at the concentration of 0.5 mM in the presence of TGase 2 (0.5 ng/ml) or Factor XIIIa (5 ng/ml) in TBS buffer containing 5 mM CaCl2 and 1 mM DTT at 37 °C. At the indicated times, 50 μl of the reaction mixture was transferred to a microtiter 96-well plate (Maxi-sorp; Nunc) containing 50 μl of ice-cold 0.5 M EDTA. After reaction, the 96-well plate was incubated for 1 h at 37 °C so that casein in the reaction mixture was attached to each well. The uncoated sites were blocked with skim milk in 0.1 M Tris-HCl (pH 8.0) (Tris buffer). Each well was then washed once with Tris buffer alone. Diluted streptavidin peroxidase (Rockland) was added to each well and incubated for 1 h at 37 °C. The wells were washed with Tris buffer containing 0.01% Triton X-100 once and then Tris buffer four times. Conjugation of streptavidin peroxidase into biotinylated peptide was measured by addition of H2O2 and O-phenylenediamine. An equal volume of 2.5 N H2SO4 was added, and the absorbance at 450 nm was measured.

Analysis of Inhibitory Effect of Synthetic Peptides on Cross-linking Reaction—Bovine casein and human fibrinogen (Calbiochem) were used as appropriate substrates for TGase 2 and Factor XIIIa, respectively. The latter case, bovine thrombin was co-incubated in the reaction mixture. Each substrate protein (200 ng/ml) was incubated with TGase 2 (0.5 ng/ml) or Factor XIII (5 ng/ml) in TBS buffer containing 1 mM DTT and 5 mM CaCl2 in the presence or absence of the peptides pepT26 and pepF11KA. The reaction products were subjected to SDS-PAGE followed by Coomassie Brilliant Blue (CBB) staining.

RESULTS

Establishment of a Screening System Using a Phage Display Random Peptide Library—A total of 1.5 × 1011 phage clones (displaying 12-mer peptides) were incubated with Bio-Cd in the presence of TGase 2 or Factor XIIIa. Following catalytic reactions, phage clones that had bound Bio-Cd covalently were selected by avidin affinity purification. These phages were subjected to four additional transamidation reaction/selec-
tion/amplification cycles. The TGase reaction time was shortened gradually with each amplification to increase the stringency of selection. Among the clones selected through multiple rounds, 40 and 35 phage clones were chosen arbitrarily from reactions of TGase 2 and Factor XIIIa, respectively. The nucleotide sequence encoding the peptide displayed by each clone was determined, and the amino acid sequences were deduced (Fig. 1, A and B).

List of Identified Preferred Sequences—Sequence analysis indicated that 39 of the 40 clones selected in the TGase 2 screening contained a glutamine residue close to the N terminus. The clone that did not contain a glutamine residue was used as a negative control (Ctrl in Figs. 2 and 3).

FIGURE 1. Alignment of preferred substrate peptide sequences for the phage clones selected. Amino acid sequences of peptides displayed on the phage clones that were selected by screening with TGase 2 (A) and Factor XIIIa (B) are aligned according to the potential reactive glutamine residues. Glutamines and commonly observed amino acid residues are shaded. The hydrophobic amino acid in the +3 position is heavily shaded. Each clone marked with an asterisk was evaluated as a GST(QN) fusion protein using the Dansyl-Cd incorporation assay (see Figs. 2 and 3).
Peptide sequences were aligned in relation to the potential reactive glutamine residue. Half of the clones were found to display sequences containing QxP (where x represents a non-conserved amino acid). The amino acid sequences obtained through the selection were divided into four groups: QxP/H9278D(P) (where /H9278 represents a hydrophobic amino acid), QxP/H9278, Qxx/H9278D(P), and sequences with no apparent motif.

In reactions with Factor XIIIa, 28 of 35 clones selected contained glutamine residues, most of which were located in the second position from the N terminus. Alignment of the 28 clones resulted in division of the preferred peptide sequences into three groups. Half of the clones included the sequence Qxx/H9278WP, whereas the remainder contained Qxx/φ or no biased sequences.

Evaluation of Preferred Sequences Using Recombinant Proteins—To evaluate the peptide sequences as glutamine donor substrates, we used labeled primary amine in the presence of TGase 2 or Factor XIIIa and measured the amount of binding to the glutamine residue. Dansyl-Cd, a fluorescence-labeled pentylamine, is widely used for transamidation activity assays. Its incorporation into substrate proteins separated by SDS-PAGE can be visualized using UV irradiation. We were unable to detect incorporation of Dansyl-Cd into phage particles, possibly because of the small amount of substrate peptide available for the reaction (data not shown). Therefore, the peptide sequences were produced in E. coli as recombinant GST fusion proteins. Because small amounts of Dansyl-Cd were incorporated into GST, we modified the GST to reduce its reaction with the primary amine. DNA substitutions were performed so that all glutamine residues (Gln-15, -67, -188, -204, and -207) present in GST were substituted with asparagines prior to construction of the expression vector. Consequently, the fusion proteins contained a modified GST (GST(QN)), as well as a hexahistidine tag at the C terminus (Fig. 2A).

For analyses of the peptide-GST(QN) fusion proteins, sequences were selected from at least each group of motifs. Recombinant fusion proteins that were expressed and purified (Fig. 1, A and B, marked with an asterisk) were then incubated with Dansyl-Cd in the presence of Factor XIIIa, 28 of 35 clones selected contained glutamine residues, most of which were located in the second position from the N terminus.
Screening for the Preferred Sequence of Transglutaminase

Peptide-GST(QN) fusion proteins were reacted with Dansyl-Cd for 10 min in the presence of TGase 2 (A) or Factor XIIIa (B). Analysis of the reaction products was performed as described in Fig. 2. The intensity of fluorescence of each product on the SDS-PAGE gel is represented by a column on the graph. Fluorescence intensity of the reaction products using DMC as substrate is expressed as a standard value (1.0). Ctrl is as described in Fig. 2.

Figure 4. Cross-reactivity of preferred sequences with TGase 2 and Factor XIIIa. Peptide-GST(QN) fusion proteins were reacted with Dansyl-Cd for 10 min in the presence of TGase 2 (A) or Factor XIIIa (B). Analysis of the reaction products was performed as described in Fig. 2. The intensity of fluorescence of each product on the SDS-PAGE gel is represented by a column on the graph. Fluorescence intensity of the reaction products using DMC as substrate is expressed as a standard value (1.0). Ctrl is as described in Fig. 2.

Either TGase 2 or Factor XIIIa. The time-dependent reaction products were separated by SDS-PAGE, visualized under UV illumination, and then aligned (Fig. 2, TGase 2; Fig. 3, Factor XIIIa). Fluorescence intensity was measured and evaluated (Figs. 2C and 3B). In both transamidation reactions, DMC was used as a positive control, and the 10-min reaction products were used for standard values. The GST(QN) fusion proteins that were obtained in screening in the TGase 2 reactions and that did not contain any glutamine residues were used as a negative control (Figs. 2–6, Ctrl).

In TGase 2 reactions, the level of incorporation of Dansyl-Cd into the fusion proteins increased with incubation time (Fig. 2). Comparison of the levels of incorporation between GST(QN) fusion proteins indicated that T1-GST(QN), T5-GST(QN), and T8-GST(QN) incorporated less Dansyl-Cd than either T26-GST(QN) or T29-GST(QN), which reacted strongly.

In contrast, in the reaction with Factor XIIIa, most GST(QN) fusion proteins with selected sequences showed significant levels (Fig. 3). Fused proteins of F6, -11, -12, -13, -16, and -19, which belong to the major group (motif: QxxdxxWP), showed apparent reactivity. Among them, F11-GST(QN) exhibited the greatest level. Although F17 and F28 sequences partly coincided with the sequence motif in the major group, both also showed considerable levels.

Evaluation of Specificities for TGase 2 and Factor XIIIa—To evaluate isozyme specificity, the GST(QN) fusion proteins were reacted in the presence of TGase 2 or Factor XIIIa. After a 10-min incubation, the reaction products were analyzed (Fig. 4, A and B, respectively). Generally, most peptide-GST(QN) fusion proteins designed from substrate sequences preferred by TGase 2 exhibited a greater incorporation of Dansyl-Cd when the reactions were catalyzed by TGase 2 rather than by Factor XIIIa. In particular, T26-GST(QN), T29-GST(QN), and T32-GST(QN) displayed higher specificities for each enzyme reaction. In contrast, several GST(QN) fusion proteins containing sequences preferred by Factor XIIIa showed a similar level of incorporation, regardless of which isozyme catalyzed the reaction. F11-GST(QN), F16-GST(QN), and F19-GST(QN) exhibited the highest specificity in the reaction with Factor XIIIa.

To examine the specificity of the preferred sequences for each isozyme, two sequences (T26 and F11) were selected and investigated using radioactive polyamine ([3H]putrescine) in the presence of TGase 2 or Factor XIIIa (Fig. 5). TGase 2 catalyzed the incorporation of putrescine into T26-GST(QN), but not F11-GST(QN), whereas the reverse was observed for reactions catalyzed by Factor XIIIa. These results suggest both sequences have significant specificity in the transamidation reaction.

Comparison with Glutamine Donor Sequences from Previously Reported Substrate Proteins—Several sequences have previously been reported as glutamine donor substrates. To compare known substrates with the sequences identified in this study, GST(QN) fusion proteins containing several sequences at the N terminus were produced and analyzed: α,-plasmin inhibitor (NQQVQSPVTLGKL), fibronectin (QQIV, PGGQQIV) trappin (GQDPVK), substance P (RPKQQQFGLM), αβ-crystallin (TVQQEL), and β-endorphin (TSEKSGTPLVT). Expectedly, most fusion proteins showed favorable reactivity in the catalytic reaction by TGase 2, whereas fusion proteins with sequences from α- plasmin inhibitor reacted by Factor XIIIa. However, compared with T26-GST(QN) and F11-GST(QN), these fusion proteins, respectively, exhibited much higher reactivity in TGase 2 or Factor XIIIa reactions (Fig. 6).

Evaluation of Synthetic Peptides—To investigate the reactivity and specificity of sequences T26 and F11, we examined the activities toward lysine donor proteins using synthetic peptides (pepT26 and pepF11KA). The lysine residue in F11 was substituted with alanine to prevent self cross-linking. Biotin-labeled peptides were reacted with casein in an assay system using a microtiter plate. Time-dependent incorporations of pepT26 and pepF11KA were observed, demonstrating that these sequences can also act as glutamine donor substrates in a
peptide form (Fig. 7). Consistent with the results obtained using the GST(QN) fusion proteins, pepT26 exhibited greater levels of incorporation into the casein than pepF11KA in TGase 2 catalytic reactions and pepF11KA displayed greater incorporation than pepT26 in reactions catalyzed by Factor XIIIa.

As pepT26 or pepF11KA might represent possible competitive inhibitors for TGase substrates, both were co-incubated in casein and fibrin cross-linking reactions with TGase 2 and Factor XIIIa (Fig. 8, A and B, respectively). In the absence of a competing peptide, cross-linked molecules of casein resulted in large unique bands, which did not occur in the presence of EDTA. In the presence of pepT26, the cross-linked casein products decreased and the uncrossed casein increased in a concentration-dependent manner, whereas the presence of pepF11KA did not affect the reaction (Fig. 8A). In the absence of any competing peptide, Factor XIIIa catalyzed the reduction of monomeric fibrin γ-chains, resulting in the production of α-polymers and γ-γ chains, which did not occur in the presence of EDTA (Fig. 8B). Concentration-dependent inhibition of cross-linking reactions was observed between 0.05 and 0.5 mM pepT26, and slight inhibition was observed with 0.5 mM pepF11KA. However, when the amounts of uncrossed and cross-linked γ-chain reaction products were compared, the inhibition effected by pepT26 was less than that of F11KA.

**DISCUSSION**

The precise mechanism governing recognition of target substrates by TGases remains unclear. It is evident that there are essential primary structures for interactions between the enzyme and glutamine residues in the substrate, although their secondary and tertiary structures are important factors (5, 23, 33). Structural analysis has demonstrated that the relative positions of residues in the substrate-binding site are highly conserved in TGases (5). However, charge distribution differs among the various isozymes, and it is assumed that each has unique preferred substrate sequences. Thus far, analyses of information in the TGase substrate data base have indicated several sequences that appear to be favored in these enzymatic reactions (20). However, there has been no reported use of an unbiased random peptide library to screen for preferred substrate sequences.

Phage display has been used to identify functional peptides based on the results of interacting sequences to various enzymes. It might be possible to identify preferred sequences through direct interaction between the TGase and displayed peptide on phage. In our established system, however, we took advantage of displayed peptides using them as substrates for incorporation of labeled primary amine. First, we demonstrated that the modified phage display system successfully determined the substrate sequence preferred by TGase 2 and Factor XIIIa.

In screening, we performed five rounds of amplification using the incorporation of Bio-Cd and affinity purification. Although several clones were analyzed through two additional rounds of amplification, their sequences exhibited a group distribution similar to the motifs obtained through five rounds (data not shown). In addition, several clones with identical sequences were obtained among eluted phages through avidin affinity purification. Therefore, we considered that five rounds were sufficient enrichment for selection of the phage clones displaying preferred sequences.

As expected, identification and analysis of the obtained sequences indicated that the preferred sequences were unique to TGase 2 and Factor XIIIa. This fact suggests that other members of the TGase family might also exhibit distinct substrate primary sequence preferences. In addition, it appears that reactive glutamine residues are commonly located close to the N terminus, although the sequences preferred by each isozyme differ. This finding is consistent with previous reports that describe an N-terminal biased for reactive glutamine residues (5, 15, 18). In the obtained clones, sequences displaying no glutamine residues were obtained. Because avidin has been reported to have affinity to some peptide sequences, there might be false-positive phage clones remaining during purification (34).

Studies of celiac disease have identified highly reactive glutamine residues for TGase 2 in searches for preferred deamidation sites of...
glutamine residue. In this study, Qxx
phobic amino acids were commonly observed at position
study. The difference from the glutamine residue, respectively, was first identified in this
preference for asparagine and proline, located at the
recognized by TGase 2 in transamidation or deamidation. Additionally, a
preferred and contained QxY, suggesting that this sequence is also rec-
deamidation sequences. As a new finding, both T26 and T29 were highly
Taken together, our study demonstrated that phage display screening is
inhibition by the selected peptides on cross-linking reactions of TGase
2 and Factor XIIIa. Cross-linking reactions of casein (A) and fibrinogen (B) were
performed in the presence of TGase 2 for 30 min and Factor XIIIa for 10 min, respec-
tively. pepT26 and pepF11KA (0, 0.01, 0.05, 0.1, and 0.5 mM) were added to the
reaction mixtures. A reaction performed in the absence of peptides and the presence of 5 mM EDTA was loaded in the
left lane. Me2SO was used as solvent for the peptides and was present at 1% in all reaction mixtures. The reaction products were analyzed by 12.5% (A) and 7.5% (B) SDS-PAGE followed by CBB staining.

were various in some sequences that do not correspond to the major
sequence motifs. Additional unknown rules to enhance or decrease the
reactivity in sequences might exist. In addition, the maximum values for
incorporating Dansyl-Cd were different despite similar initial rates of
incorporation. The precise reason is uncertain, but it might reflect the
difference in the degree of transamidation/deamidation in each
sequence in addition to reactivity. Among the amino acid sequences
examined, two (T26 and F11) were identified as highest, isozyme-spe-
cific substrates for TGase 2 and Factor XIIIa, respectively.

In addition, several sequences exhibited unique reactivities (Fig. 4).
For example, T1 showed the opposite reactions with TGase 2 and Factor
XIIIa; T8, T16, and T20 were specific for TGase 2 cross-linking and
displayed less reactivity with Factor XIIIa. Although we selected two
representative sequences (T26 and F11) for further analysis as peptides
in this study, the other sequences might also contribute to our eventual
understanding of recognition mechanisms. Furthermore, fusion pro-
teins with these sequences appeared to be superior to those with previ-
ously identified glutamine donor sequences (Fig. 6), suggesting various
applications of both peptide sequences might be possible as the TGase
substrate.

Knowledge of sequence specificity can also facilitate the design and
optimization of potent and selective inhibitors. We used substrate
competition to examine whether the selected peptides were potential inhibi-
tors for cross-linking reactions by TGases. pepF11KA exhibited spe-
cific inhibition of the Factor XIIIa cross-linking reaction, whereas it did
not affect the TGase 2 reaction. Although pepT26 showed specific and
considerable blocking activity against cross-linking reactions in TGase
2, it inhibited Factor XIIIa reaction only slightly at the higher concen-
tration (0.5 mM). Possibly, because the peptide was smaller than the
fusion protein, it was able to interact with Factor XIIIa at higher con-
centration. Therefore, modification of these peptides might provide
inhibitors with improved specificity and potency. Thus far, several
inhibitory peptides of Factor XIIIa and TGase 2 activities have been
identified based on partial amino acid sequences of substrates (10–12,
38). Both pepT26 and pepF11KA are not highly homologous to the
previously identified sequences, but their sequences exhibited superior
reactivities as glutamine donor, suggesting that both peptides have
more prominent inhibitory effects on the cross-linking reactions.
Therefore, both peptides might feature as future research tools in inves-
tigation of cellular function and substrate recognition mechanisms of
TGases.

In conclusion, using a random peptide library, we established a
screening system for identification of specific amino acid sequences
preferred by TGase isozymes. This screening system is now available for
analyses of other mammalian TGase isozymes and bacterial transglu-
taminases and may prove useful in investigation of respective substrates
and reaction mechanisms of members of the TGase family. For ex-
ample, this system might help to elucidate the cooperative roles played by
TGases 1, 3, and 5 in formation of a cornified envelop in skin epidermis,
which occurs by cross-linking various structural proteins such as lori-
crin, involucrin, and small proline-rich proteins (39–41). In addition,
using this system for the investigation of substrate specificity might
provide a basis for the design of specific inhibitors and will form the
subject of future studies.

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