Naturally Occurring Epsilon Gamma Glutamyl Lysine Isopeptide Crosslinks in Human Neuroblastoma SH-SY5Y Cells

Oksana Lockridge* and Lawrence M. Schopfer

ABSTRACT: Zero-length isopeptide crosslinks between the side chains of glutamine and lysine are the product of transglutaminase activity. It is generally accepted that transglutaminase activity is dormant under physiological conditions because the calcium concentration inside cells is too low to activate transglutaminase to an open conformation with access to the catalytic triad. Traditional assays for transglutaminase activity measure incorporation of biotin pentylamine or of radiolabeled putrescine in the presence of added calcium. In this report, we identified naturally occurring isopeptide crosslinked proteins using the following steps: immunopurification of tryptic peptides by binding to anti-isopeptide antibody 81D1C2, separation of immunopurified peptides by liquid chromatography–tandem mass spectrometry, Protein Prospector database searches of mass spectrometry data for isopeptide crosslinked peptides, and manual evaluation of candidate crosslinked peptide pairs. The most labor intensive step was manual evaluation. We developed criteria for accepting and rejecting candidate crosslinked peptides and showed examples of MS/MS spectra that confirm or invalidate a possible crosslink. The SH-SY5Y cells that we examined for crosslinked proteins had not been exposed to calcium and had been lysed in the presence of ethylenediaminetetraacetic acid. This precaution allows us to claim that the crosslinks we found inside the cells occurred naturally under physiological conditions. The quantity of crosslinks was very low, and the crosslinked proteins were mostly low abundance proteins. In conclusion, intracellular transglutaminase crosslinking/transamidase activity is very low but detectable. The low level of intracellular crosslinked proteins is consistent with tight regulation of transglutaminase activity.

INTRODUCTION

Zero-length isopeptide crosslinks between the side chains of lysine and glutamine are the product of transglutaminase activity.1−3 Transglutaminase (TG2) activity is essential for neurite outgrowth in human neuroblastoma cells.4 Knockdown of transglutaminase 2 in primary cortical neurons decreased the neurite outgrowth in human neuroblastoma cells.4 Knockdown cell carcinoma, and gastric cancers.17

Isopeptide bonds stabilize blood clots, skin, hair, and the protective mucus network lining the gastrointestinal tract.8 Excessive or inappropriate isopeptide bond formation promotes polymerization of proteins to high molecular weight aggregates implicated in Alzheimer’s disease (AD), Huntington’s chorea, Parkinson’s disease, and Lewy body disease.9−11 Transglutaminase-mediated crosslinking of proteins in the eye results in cataracts.12 Deamidation of gluten peptides by transglutaminase leads to celiac disease.13,14 Cancer stem cells that overexpress transglutaminase are associated with metastatic spread and drug resistance.15,16 Transglutaminase has been proposed as a target for treatment of mesothelioma, renal cell carcinoma, and gastric cancers.17−19

Intracellular transglutaminase adopts a closed conformation that blocks access to the catalytic triad Cys277, His335, and Asp358.20 Therefore, intracellular TG2 is generally assumed to have minimal or no crosslinking activity. (See the Discussion section.) Our finding of KQ crosslinked peptides inside cells supports the conclusion that intracellular TG2 crosslinking activity is not zero.

Established methods to identify protein targets of transglutaminase crosslinking activity include labeling with fluorescent dansyl or biotinylated probes21 followed by mass spectrometry analysis22,23 or by incorporation of radiolabeled putrescine followed by immunoblotting.24,25 Our mass spectrometry method builds on the work of Nemes et al. who identified crosslinked proteins in the brains of AD and Lewy body disease patients by immunopurifying peptides containing an isopeptide bond, followed by mass spectrometry.9,10

In this report, we used mass spectrometry, Protein Prospector database searches, and manual evaluation to identify naturally occurring crosslinked peptides in human neuroblastoma SH-SY5Y cells. Manual evaluation was a critical step for identifying crosslinked peptides. Our goal in this report is to establish criteria for accepting and rejecting

Received: April 21, 2022
Accepted: June 7, 2022
Published: June 16, 2022
candidate crosslinked peptide pairs in a complex protein mixture.

**MATERIALS**

Human neuroblastoma SH-SY5Y cells (ATCC CRL-2266) were grown in DMEM/F12 GlutaMAX (Gibco 10565-018) supplemented with fetal bovine serum (Life Tech 16000044) and penicillin & streptomycin (Gibco 15140-122). Cells were differentiated in a serum-free medium containing trans-retinoic acid. Cells were lysed in Pierce IP Durapore spin filters (Thermo Scientific 877877). Cells were subjected to liquid chromatography tandem mass spectrometry. The antibody isopeptide monoclonal 81D1C2 at room temperature for 8 h. Tryptic peptides were complexed with mouse anti-isopeptide antibody 81D1C2, and the beads and liquid were transferred to a 0.45 μm filter. The beads and liquid were transferred to a new microtube. Bound peptides were released from the washed beads by incubating the basket of beads with 0.1 mL of 50% acetonitrile and 1% formic acid for 0.5 to 1 h at room temperature. The released peptides were collected in the flow through by brief centrifugation. The extraction step was repeated twice. The combined flow through was dried by vacuum centrifugation.

**Sample Preparation for Mass Spectrometry.** The dry sample was dissolved in 20 μL of water. The sample was centrifuged for 30 min at 14,000 × g and 4 °C. The top ten microliters were transferred to an autosampler vial.

**Liquid Chromatography—Tandem Mass Spectrometry.** Peptide separation was performed with a Thermo RS LC Ultimate 3000 ultrahigh pressure liquid chromatography system (Thermo Scientific) at 36 °C. Solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in 80% acetonitrile. Peptides were loaded onto an Acclaim PepMap 100 C18 trap column (75 μm × 2 cm; Thermo Scientific cat# 165535) at a flow rate of 4 μL/min and washed with 98% solvent A/2% solvent B for 10 min. Then, they were transferred to a Thermo Easy-Spray PepMap RS LC18 column (75 μm × 50 cm with 2 μm particles; Thermo Scientific cat# ES803) and separated at a flow rate of 300 μL/min using a gradient of 9 to 50% solvent B in 30 min, 50 to 99% solvent B in 40 min, hold at 99% solvent B for 10 min, 99 to 9% solvent B in 4 min, and hold at 9% solvent B for 16 min. Eluted peptides were sprayed directly into a Thermo Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific). Data were collected using data dependent acquisition. A survey full scan MS (from 350 to 1800 m/z) was acquired in the Orbitrap with a resolution of 120,000. The AGC target (Automatic Gain Control for setting the ion population in the Orbitrap before collecting the MS) was set at 4 × 10^6, and the ion filling time was set at 50 ms. The 25 most intense ions with a charge state of 2−6 were isolated in a 3 s cycle and fragmented using high-energy collision-induced dissociation with 35% normalized collision energy. Fragment ions were detected in the Orbitrap with a mass resolution of 30,000 at 200 m/z. The AGC target for MS/MS was set at 5 × 10^5, and dynamic exclusion was set at 30 s with a 10 ppm mass window. Data were reported in *.raw format. The *.raw data files were converted to *.mgf files using MSConvert (ProteoWizard Tools from SourceForge).

**Protein Prospector Search for Crosslinked Peptides.** The search parameters on the Batch-Tag Web page in Protein Prospector (prospector.ucsf.edu/prospector/mshome.htm) were as follows. (1) Database: SwissProt.2020.09.02. (2) Taxonomy: Homo sapiens. (3) Precursor charge range: 2, 3, 4, and 5. (4) Parent Tol 20 ppm, Frag Tol 30 ppm. (5) Digest: trypsin. (6) Max missed cleavages: 3. (7) Constant mods: none selected because the proteins were not reduced and alkylated. (8) Expectation calc method: none. (9) Variable mods: oxidation (M). (10) User-defined variable modifications were left blank. (11) Mass modifications at default setting 18 to 3883 Da. (12) Checkmark in the boxes for K and Q. (13) Checkmark in the box Unchanged avoids reporting peptides with maximized recovery because beads were not lost in the wash steps. Beads were washed 5 times with 0.4 mL of RIPA buffer (25 mM Tris−HCl pH 7.6, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl) followed by 5 washes with water. Salts and detergents were washed off with water. The flow through in each wash step was discarded.

# Techniques

**Cell Lysis and Protein Concentration.** Cells were grown in DMEM/F12 GlutaMAX (Gibco 10565-018) supplemented with fetal bovine serum, penicillin, and streptomycin, in a humidified 5% carbon dioxide incubator at 37 °C. After 5 days, when cells were 70−80% confluent, cells were washed with phosphate buffered saline (PBS) and harvested. Another set of 70−80% confluent SH-SY5Y cells was incubated in DMEM/F12 GlutaMAX (no serum) supplemented with 10 μM trans-retinoic acid and 10 μM dichlorvos. After 2 days, cells were harvested from seven T75 flasks. Cytoplasmic transglutaminase protein is upregulated in the differentiated in a serum-free medium containing trans-retinoic acid. After 5 days, the top ten microliters were transferred to an autosampler vial.

**Trypsin Digestion.** The cell lysate supernatant containing 200 μg of protein was diluted with 20 mM ammonium bicarbonate pH 8 to 200 μL. Proteins were denatured in a boiling water bath for 3 min. The denatured proteins were digested with 4 μg of trypsin (8 μL) at 37 °C for 16 h. Trypsin was inactivated by heating the digest in a boiling water bath for 3 min.

**Immunopurification of Tryptic Peptides.** The heat-treated digest was incubated with 8 μg (8 μL) of anti-isopeptide monoclonal 81D1C2 at room temperature for 8 h. The antibody−peptide complexes were immobilized by adding 0.1 mL of a 1:1 suspension of Protein Gagarose beads in PBS. The sample was rotated overnight at room temperature.

The beads and liquid were transferred to a 0.45 μm Durapore spin filter (Millipore UFC30HV00). Use of the spin filter maximized recovery because beads were not lost in the wash steps. Beads were washed 5 times with 0.4 mL of RIPA buffer (25 mM Tris−HCl pH 7.6, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl) followed by 5 washes with water. Salts and detergents were washed off with water. The flow through in each wash step was discarded.

The basket of washed beads was transferred to a new microtube. Bound peptides were released from the washed beads by incubating the basket of beads with 0.1 mL of 50% acetonitrile and 1% formic acid for 0.5 to 1 h at room temperature. The released peptides were collected in the flow through by brief centrifugation. The extraction step was repeated twice. The combined flow through was dried by vacuum centrifugation.
pattern for the crosslinked peptides listed in Table 1. The ppm, retention time, and Score and Score difference. A link to the MS/MS spectrum showed the % match between ions assigned to the crosslinked peptide and total ions in the spectrum. A Table of Peak Matches linked to each MS/MS spectrum listed the mass of each peak, the ion type (y, b, a), blank for peaks that did not fit the crosslinked peptide, the charge of each ion, and mass error. We used the Table of Peak Matches to calculate the number of crosslink-specific ions for each candidate crosslinked peptide. A crosslink-specific ion contains a fragment from one peptide plus the entire second peptide. We selected a preliminary set of crosslinked peptides for further evaluation based on a Score + Score difference greater than 30, matched intensity greater than 40%, and a minimum of two crosslink specific ions in a series that defined an amino acid.

Manual Evaluation. Manual evaluation of each MS/MS spectrum started with assigning the charge state of each peak in the MS/MS spectrum retrieved from Thermo Scientific Xcalibur/Qual Browser. Protein Prospector did not give the charge state of peaks that did not fit the candidate crosslinked peptide. Therefore, the charge state was obtained from Qual Browser files. The monoisotopic mass of each peak was determined. The interval between peaks, grouped by the charge state, was calculated and correlated with the mass of an amino acid.

## RESULTS

Transglutaminase makes a covalent bond between the side chains of glutamine and lysine to create an epsilon (gamma-glutamyl) lysine isopeptide bond with release of ammonia. See Figure 1.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Transglutaminase (TG) catalyzes the formation of a covalent bond between the side chains of glutamine and lysine in proteins. The isopeptide bond is resistant to trypsin.

The MS/MS spectrum in Figure 2 is a typical fragmentation pattern for the crosslinked peptides listed in Table 1. The peptides crosslinked in Figure 2 were identified in SH-SYSY cells that had been treated with retinoic acid and lysed in the presence of EDTA without added calcium chloride. The absence of calcium chloride is emphasized because transglutaminase crosslinking activity is expected to occur only in the presence of calcium chloride concentrations high enough to change the TG2 protein conformation from closed to open. The intracellular calcium chloride concentration is estimated to be too low at 100 nM to activate transglutaminase to the open conformation that has crosslinking activity. Despite the low intracellular calcium concentration, TG2-catalyzed crosslinking activity is detectable by mass spectrometry of immunopurified crosslinked peptides.

The MS/MS spectrum in Figure 2 fulfills our criteria for a crosslinked peptide pair. The required features are (1) the presence of a minimum of two crosslink specific ions that define an amino acid. The definition of a crosslink specific ion is an ion that contains residues from both peptides. In Figure 2, the crosslink specific ions are green y7+3, y8+3, and y9+3, blue y7+3, y8+3, and y9+3, and blue y8+4 and y9+4. The structures of green crosslink specific ions y7+3, y8+3, and y9+3 give a visual definition of the term “crosslink specific ion” as ions that contain residues from both peptides. The structures of blue y8+4 and y9+4 ions demonstrate the meaning of the term “ladder ions” as ions that have the same charge as the parent ion, though they have lost residues from the N-terminal of one peptide.

Catalyst

- **Results**
- **Figure 2.** MS/MS spectrum showing lysine 109 in the green peptide (Q8WU55). GATA zinc finger domain-containing protein 1. GATD1 crosslinked to glutamine 230 in the blue peptide (P35711). Transcription factor, SOX5) via a zero-length isopeptide bond. The parent ion MH+4 is at 586.32 m/z. Crosslink specific ions are green y7+3, y8+3, and y9+3, blue y7+3, y8+3, and y9+3, and blue y8+4 and y9+4. The structures of green crosslink specific ions y7+3, y8+3, and y9+3 give a visual definition of the term “crosslink specific ion” as ions that contain residues from both peptides. The structures of blue y8+4 and y9+4 ions demonstrate the meaning of the term “ladder ions” as ions that have the same charge as the parent ion, though they have lost residues from the N-terminal of one peptide.
| #  | Z Score | Score diff | % match | crosslinked peptides with linked amino acids numbered | UniProt short name |
|----|---------|------------|---------|-----------------------------------------------------|------------------|
| 1  | +3      | 43.8       | 67.2    | GKVRVEK_{193,194,195}                               | Q01484 ANK2      |
| 2  | +3      | 40.2       | 54.3    | EKK_{193,194,195}                                 | Q9N571 GKN1      |
| 3  | +3      | 30.9       | 60.5    | SAEC_{89,90,91}                                    | Q9NP80 PLPL8     |
| 4  | +3      | 30.7       | 43.5    | TTVKVPKG_{195,196}                                 | Q99333 BAG1      |
| 5  | +4      | 55.5       | 74.8    | KK_{241,242,243}                                    | Q91772 SPEG      |
| 6  | +4      | 52.2       | 79.5    | KVVRNLVQK_{294,295}                                | Q9HCC0 MCCB      |
| 7  | +4      | 43.7       | 50.0    | YK_{338,339,340}                                    | Q15772 S271      |
| 8  | +4      | 42.1       | 73.5    | K_{48,49,50}                                        | Q16181 SEPT7     |
| 9  | +4      | 38.2       | 59.5    | REHVAK_{194,195,196}                                | Q10809 CH60      |
| 10 | +4      | 38.0       | 57.1    | MVVSAIVDTLQ_{195,196}                               | Q92543 SNX19     |
| 11 | +4      | 37.9       | 61.3    | YNVRIQ_{195,196}                                    | Q95271 TNLK      |
| 12 | +4      | 36.0       | 58.5    | RGNNGDRVSLJK_{195,196}                              | Q75Q2N1 INT8     |
| 13 | +4      | 32.5       | 70.3    | REK_{191,192,193}                                   | Q14181 PSA7      |
| 14 | +5      | 64.2       | 70.8    | QSK_{198,199,200}                                   | Q936R78 GCP5     |
| 15 | +5      | 56.0       | 61.6    | ELRNFSRLSIALQ_{192,193}                             | Q9M3P71 SOX5     |
| 16 | +5      | 48.7       | 75.2    | ALELRVLQ_{195,196}                                  | Q10809 CH60      |
| 17 | +5      | 46.6       | 81.9    | K_{193,194,195}                                     | Q13846 DNA11     |
| 18 | +5      | 45.9       | 81.1    | KGQEVQK_{195,196}                                   | Q9Q416 DNA11     |
| 19 | +5      | 42.5       | 67.3    | LLEAEK_{226,227,228}                                | Q98999 RBL2      |
| 20 | +5      | 39.9       | 48.0    | LLLEAEK_{226,227,228}                               | Q98999 RBL2      |
| 21 | +5      | 38.4       | 48.9    | ISAFPLARQLW_{193,194}                                | Q98999 RBL2      |
| 22 | +5      | 36.1       | 47.6    | DK_{189,190,191}                                    | Q98999 RBL2      |
| 23 | +5      | 35.9       | 53.7    | TNLQPAKQNSLMoxK_{101,102}                           | Q8P2E3 ZFEN1     |
| 24 | +5      | 35.8       | 55.5    | IQQSAGELATSQ_{186,187}                              | Q98P2E3 ZFEN1     |
| 25 | +5      | 35.7       | 51.7    | ELFKQK_{186,187}                                    | Q98P2E3 ZFEN1     |
| 26 | +5      | 35.6       | 53.6    | KKSSEXKPTSLGLAGGHK_{206,207}EKR                      | Q986R0 CIC       |
| 27 | +5      | 35.2       | 61.7    | SWSLJ_{207,208,209}                                 | Q986R0 CIC       |
| 28 | +4      | 35.0       | 52.1    | LLQKAYAQG_{190,191}                                 | Q99173 DCP2      |
| 29 | +4      | 32.1       | 49.4    | EIFDK_{138,139,140}                                 | Q99173 DCP2      |
| 30 | +4      | 30.9       | 59.7    | ASLC_{236,237,238}                                  | Q9Y2X0 MED16     |
| 31 | +5      | 28.4       | 45.2    | SLMAGK_{163,164,165}                                | Q9UBF8 PI4KB     |
| 32 | +5      | 28.4       | 45.2    | SLMAGK_{163,164,165}                                | Q9UBF8 PI4KB     |
blue ion at 850.49. It lost L from the blue peptide to yield the 793.95 y3+2 blue ion. The 793.95 ion lost R from the C-terminus of the green peptide to yield 715.90, which lost E from the green peptide to yield 651.38. The 651.38 ion lost K from the C-terminus of the blue peptide to yield the 587.33 ion. The 587.33 ion lost D from the green peptide to yield the 520.83 ion. The structures in panel B are missing from the adjacent structure. For example, the red L in the structure at 850.49 is missing from the structure at 793.95. Black lines in the Protein Prospector spectrum are for masses the software did not assign to the crosslinked peptide. We assigned three black lines to the structures at 520.83, 587.33, and 651.38 m/z.

Another unusual fragmentation spectrum is shown in Figure 4. A series of +3 ions from 774.12 to 908.19 echoes the series of +4 ions from 670.90 to 771.45. Both series yield the sequence IAMoxxA. The +3 ion at 774.12 has lost five residues from the N-terminal of the green peptide, two residues from the C-terminal of the green peptide, and one residue from the C-terminal of the blue peptide. The 774.12 m/z ion has also lost ammonia. The structure of the 774.12 m/z ion is shown. Masses associated with the +3 series are marked by arrows in the MS/MS spectrum. The unusual +3 ion series was identified by manual evaluation. It was not identified by Protein Prospector, whereas the comparable +4 series was identified by Protein Prospector.

For comparison, the structure of the green y9+4 ion is shown so that the difference between a standard y9+4 ion and the comparable, unusual +3 fragment at 774.12 m/z can be visualized. Search programs correctly identify the fragmentation pattern for a series where one peptide remains intact, while the second peptide loses amino acids from one end, such as the +4 series. The green y9+4 ion at 670.90 m/z has an intact blue peptide linked to nine residues of the green peptide, having lost five residues from the N-terminal of the green peptide.
The dipeptides in Table 1 are naturally occurring crosslinked peptides produced by human transglutaminase activity inside human SH-SY5Y neuroblastoma cells. The cells had not been permeabilized and treated with calcium. The cell lysate had not been treated with calcium chloride.

Procedure for Evaluating Candidate Crosslinked Dipeptides.

1. The Search Compare page in Protein Prospector lists hundreds of candidate crosslinked peptides. We screened the candidates and made a list of those that have (a) Score + Score difference greater than 30 and (b) matched intensity greater than 40%. This reduced the number of candidate crosslinks to 89.

2. We examined 89 screened MS/MS spectra for crosslink specific ions. Crosslink specific ions are defined as ions that contain amino acids from both peptides. An acceptable candidate crosslink peptide was required to have a minimum of two crosslink specific ions in a series that defined an amino acid. We did not accept random crosslink specific ions. Crosslink specific ions can be b-ions, y-ions, ladder-ions, or ions from mixed fragmentation. Figure 2 shows structures of crosslink specific y-ions and ladder ions. Figure 3 shows structures of crosslink specific ions produced by mixed fragmentation. Figure 5 shows an MS/MS spectrum for a crosslinked peptide pair in which the crosslink specific ions are b-ions.

Criteria for Accepted Crosslinked Peptides.

1. The MS/MS spectrum must contain amino acid sequence information from both peptides. A sequence is defined as a series of adjacent amino acids. Sequences of masses that include the crosslink specific ion and ladder sequences, that is, neutral losses from the parent ion, need only be one amino acid long. N-terminal or C-terminal sequences or sequences from internal fragmentation such as at proline must be at least three amino acids long.

2. Each peptide in the crosslink must contain a minimum of five amino acids.

3. A minimum of two crosslink specific ions must be present in a series that defines a crosslink specific amino acid.

4. We often find a peptide sequence in the MS/MS spectrum that is unrelated to the crosslinked peptides. If the unrelated peptide sequence includes none of the crosslink specific ions, we ignore the extra peptide because the extra peptide does not invalidate the crosslinked peptide.

5. Sometimes, the green and blue ions have the same mass. For example, in Figure 4, green y10+4 and blue y11+4 ions both have a mass of 699.17 Da. The series continues having the same mass for green y11+4 and blue y12+4 ions of 716.93. However, green ions y9+4, y12+4, and y13+4 are present that have no matching blue ion. This means that the y9+4 to y13+4 series supports the green peptide but not the blue peptide.

Criteria for Rejecting a Candidate Crosslinked Dipeptide.

1. Exclude crosslinks in charge states +6 and +2.

2. A single crosslink specific ion is not sufficient evidence to support a crosslinked peptide.

3. If two or more amino acids are appended to a convincing crosslinked series, we do not accept the crosslink specific ions as real. See Figure 6.
The conclusion that TG2 has no crosslinking/transamidase activity under normal physiological conditions is challenged by the following experimental results. Fesus and Tarcsa found epsilon-(gamma-glutamyl)-lysine isodipeptides, the product of TG2 crosslinking activity, in Chinese Hamster Ovary cells under basal conditions. A fluorescence resonance energy transfer study found the open TG2 conformation beneath the cell membrane in human lung adenocarcinoma A549 cells, mouse fibroblasts 3T3, and Chinese hamster ovary cells. Yamaguchi and Wang detected incorporation of biotinylated pentamethylene in HCT116 cells, in activity assays performed in the presence of the calcium chelators EGTA and EDTA. These results support the conclusion that cytoplasmic TG2 has low but detectable crosslinking activity. Low but detectable transamidase activity, measured by incorporation of radiolabeled putrescine, was found in WI-38 fibroblasts and MDA-MB-231 cells. A cell permeable fluorescent inhibitor specific for TG2 was bound to the open conformation of TG2 inside the low Ca\(^{2+}\) intracellular environment of HUVEC and NIH3T3 cells.

These observations suggest that the low intracellular Ca\(^{2+}\) concentration is sufficient to activate TG2 in some cases or that factors other than Ca\(^{2+}\) can stimulate TG2 to acquire the open conformation associated with crosslinking activity. Interaction with proteins or lipids may activate TG2 crosslinking/transamidase activity.

Our finding of a limited number of crosslinked proteins in the cell lysate of human neuroblastoma cells is consistent with low, but real, intracellular TG2 crosslinking activity. Cells were lysed in the presence of EDTA, thus eliminating the possibility of activating transglutaminase crosslinking activity by calcium during handling. Isopeptide crosslinked proteins have a half-life of about 3 h in living cells, as measured by Fesus and Tarcsa with radioactive lysine. The observation that isopeptide crosslinked proteins do not accumulate supports our finding of a low level of crosslinked proteins inside a living cell.

**Crosslinked Proteins.** We had expected to find crosslinking between abundant intracellular proteins, for example, tubulin and actin. However, this was not the case. With one exception, the crosslinked proteins were low abundance proteins. The exception was P10809, the 60 kDa heat shock protein, an abundant protein in SH-SYSY cell lysate.

We had also expected to find internally crosslinked peptides, where the KQ crosslink is between two residues on the same protein. We rejected the few potential candidates because the difference in the parent ion mass between a linear peptide and a KQ crosslinked peptide is 1 Da. The mass spectrometer can tolerate a KQ crosslinked peptide if the R were a crosslink specific ion. Other instances where the interval mass for an amino acid is the same as that for a pair of amino acid intervals are as follows:

- **a.** R = V + G
- **b.** K = G + A
- **c.** Q = G + A
- **d.** W = G + E = A + D = S + V
- **e.** N = G + G

> **DISCUSSION**

**Intracellular Crosslinked Proteins Are Present.** The prevailing view is that intracellular TG2 has no crosslinking activity under normal physiological conditions because the cell has high GTP (around 100 \(\mu\)M) and low Ca\(^{2+}\) (around 100 nM) concentrations. At 100 \(\mu\)M, GTP binds to TG2 and stabilizes the closed form, which blocks access to the active site and therefore blocks crosslinking activity. Activation of TG2 to the open form is achieved by binding calcium. However, at 100 nM, Ca\(^{2+}\) is unable to compete with GTP. Thus, it is argued that TG2 cannot attain the open conformation capable of crosslinking proteins.

The conclusion that TG2 has no crosslinking/transamidase activity inside a cell under physiological conditions is...
as substrates for transglutaminase have been identified.\textsuperscript{34–37} Preferred glutamine donor peptides have a consensus sequence that depends on the TG isozyme. In Table S2, we compare the consensus sequences of preferred glutamine donor peptides to the glutamine donor peptides in the crosslinked proteins in SH-SY5Y cells. The glutamine donor peptides in SH-SY5Y cells have no consensus sequence.

**Limitations.** We found crosslinked peptides in SH-SY5Y cells that had been treated with 10 \(\mu\)M retinoic acid and 10 \(\mu\)M dichlorvos in the serum-free medium for 2 days before harvesting cells for our study. TG2 protein levels and transamidase activity are reported to be induced by retinoic acid in SH-SY5Y cells.\textsuperscript{25} Our retinoic acid-treated cells have been grown in 10% fetal bovine serum and had not been treated with retinoic acid. Thus, we suggest that our results have not been biased by retinoic acid.

We searched the cell lysates for soluble crosslinked proteins. We did not search for crosslinked proteins in the insoluble protein pellet. Highly crosslinked proteins are likely to form insoluble aggregates that we missed in our study of soluble proteins.

Dichlorvos is an organophosphorus pesticide that makes adducts on the side chains of tyrosine, serine, threonine,\textsuperscript{19} and possibly lysine though adducts on lysine have not been reported. Organophosphorus toxicants do not inhibit TG2 transamidase activity.\textsuperscript{38,39}

**CONCLUSIONS**

We describe a method for evaluating crosslinked proteins produced by the action of tissue transglutaminase, TG2. The method includes immunopurification of tryptic peptides, separation of peptides by liquid chromatography, acquisition of fragmentation spectra by mass spectrometry, Protein Prospector database search of MS/MS data, and manual evaluation of candidate crosslinks. Our criteria for accepting candidate crosslinks are conservative. Our method for evaluating zero-length isopeptide crosslinks will be useful for understanding the mechanism of therapeutic drugs aimed at inhibiting TG2 activity in cancer and other diseases.

**ASSOCIATED CONTENT**

 Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c02502.

(Table S1) Mass differences for dehydro-amino acids in peptide sequences and (Table S2) comparison of glutamine donor peptides (PDF)

**AUTHOR INFORMATION**

**Corresponding Author**

Oksana Lockridge — Eppley Institute, University of Nebraska Medical Center, Omaha, Nebraska 68198-5900, United States; orcid.org/0000-0002-8345-3640; Email: olockrid@unmc.edu

**Author**

Lawrence M. Schopfer — Eppley Institute, University of Nebraska Medical Center, Omaha, Nebraska 68198-5900, United States; orcid.org/0000-0002-1785-9247

Complete contact information is available at:

https://pubs.acs.org/10.1021/acsomega.2c02502

**Author Contributions**

O.L.: investigation, writing original draft. L.M.S.: formal analysis, review, and editing.

**Funding**

Supported by NIH grant 1R21ES030132-01A1 (to O.L.) and Fred & Pamela Buffet Cancer Center Support Grant P30CA036727.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

Mass spectrometry data were obtained by the Mass Spectrometry and Proteomics Core Facility at the University of Nebraska Medical Center, which is supported by state funds from the Nebraska Research Institute. Protein Prospector programs are available at no cost, https://prospectorm.ucsf.edu. Protein Prospector was developed in the University of California San Francisco Mass Spectrometry Facility, directed by Dr. Alma Burlingame, funded by the NIH National Institute for General Medical Sciences. Proteomics Toolkit http://db.systemsbiology.net:8080/proteomicstoolkit/ and Xcalibur/Qual Browser (Thermo Scientific) were used to identify ions in MS/MS spectra.

**ABBREVIATIONS**

SDS, sodium dodecyl sulfate

TG2, human transglutaminase type 2

PBS, phosphate buffered saline

FBS, fetal bovine serum

RIPA buffer, 25 mM Tris–HCl pH 7.6, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl

IP lysis buffer, 25 mM TrisCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol

SH-SY5Y cells, human neuroblastoma cells

MS/MS, tandem mass spectral fragmentation

**REFERENCES**

(1) Lorand, L.; Graham, R. M. Transglutaminases: crosslinking enzymes with pleiotropic functions. *Nat. Rev. Mol. Cell Biol.* 2003, 4, 140–156.

(2) Lorand, L.; Downey, J.; Gotoh, T.; Jacobsen, A.; Tokura, S. The transpeptidase system which crosslinks fibrin by gamma-glutamyl-epsilon-lysine bonds. *Biochem. Biophys. Res. Commun.* 1968, 31, 222–230.

(3) Folk, J. E.; Finlayson, J. S. The epsilon-(gamma-glutamyl)lysine crosslink and the catalytic role of transglutaminases. *Adv. Protein Chem.* 1977, 31, 1–133.

(4) Tucholski, J.; Lesort, M.; Johnson, G. V. Tissue transglutaminase is essential for neurite outgrowth in human neuroblastoma SH-SY5Y cells. *Neuroscience* 2001, 102, 481–491.

(5) Yunes-Medina, L.; Paciorkowski, A.; Nuzbrokh, Y.; Johnson, G. V. W. Depletion of transglutaminase 2 in neurons alters expression of extracellular matrix and signal transduction genes and compromises cell viability. *Mol. Cell Neurosci.* 2018, 86, 72–80.

(6) De Laurenzi, V.; Melino, G. Gene disruption of tissue transglutaminase. *Mol. Cell. Biol.* 2001, 21, 148–155.

(7) Nanda, N.; Ismama, S. E.; Owens, W. A.; Husain, A.; Mackay, F.; Graham, R. M. Targeted inactivation of Gh/tissue transglutaminase II. *J. Biol. Chem.* 2001, 276, 20673–20678.

(8) Recktenwald, C. V.; Hansson, G. C. The Reduction-insensitive Bonds of the MUC2 Mucin Are Isopeptide Bonds. *J. Biol. Chem.* 2016, 291, 13580–13590.
(9) Nemes, Z.; Petrovski, G.; Aerts, M.; Sergeant, K.; Devreeze, B.; Fusus, L. Transglutaminase-mediated intramolecular cross-linking of membrane-bound alpha-synuclein promotes amyloid formation in Lewy bodies. J. Biol. Chem. 2009, 284, 27252–27264.

(10) Nemes, Z.; Devreeze, B.; Steiner, P. M.; Van Beemen, J.; Fusus, L. Cross-linking of ubiquitin, HSP27, parkin, and alpha-synuclein by gamma-glutamyl-episonol-lysine bonds in Alzheimer’s neurofibrillary tangles. FASEB J. 2004, 18, 1135–1137.

(11) Wilhelmus, M. M. M.; de Jager, M.; Bakker, E. N. T. P.; Drukarb, B. Tissue transglutaminase in Alzheimer’s disease: involvement in pathogenesis and its potential as a therapeutic target. J. Alzheimer’s Dis. 2014, 42, S289–S303.

(12) Lorand, L.; Ismaa, S. E. Transglutaminase diseases: from biochemistry to the bedside. FASEB J. 2019, 33, 3–12.

(13) Lexhaller, B.; Ludwig, C.; Scherf, K. A. Identification of Isopeptides Between Human Tissue Transglutaminase and Wheat, Rye, and Barley Gluten Peptides. Sci. Rep. 2020, 10, 7426.

(14) Khosla, C. Celliac Disease: Lessons for and from Chemical Biology. ACS Chem. Biol. 2017, 12, 1455–1459.

(15) Tabolacci, C.; De Martin, A.; Mischiati, C.; Feriotto, G.; Beninati, S. The Role of Tissue Transglutaminase in Cancer Cell Initiation, Survival and Progression. Med. Sci. 2019, 7, 19.

(16) Eckert, R. L. Transglutaminase 2 takes center stage as a cancer cell survival factor and therapy target. Mol. Carcinog. 2019, 58, 837–853.

(17) Kim, S.-Y.; Keillor, J. W. A Precision Strategy to Cure Renal Cell Carcinoma by Targeting Transglutaminase 2. Int. J. Mol. Sci. 2020, 21, 2493.

(18) Adhikary, G.; Grun, D.; Alexander, H. R.; Friedberg, J. S.; Xu, W.; Keillor, J. W.; Kandasamy, S.; Eckert, R. L. Transglutaminase is a mesotheloma cancer stem cell survival protein that is required for tumor formation. Oncotarget 2018, 9, 34495–34505.

(19) Cho, S. Y.; Oh, Y.; Jeong, E. M.; Park, S.; Lee, D.; Wang, X.; Zeng, Q.; Qin, H.; Hu, F.; Gong, H.; Liu, X.; Zhang, G.; Na, D.; Lee, J.; Chae, J.; Suh, Y. S.; Kong, S. H.; Lee, H. J.; Kim, J. I.; Park, H.; Zhang, C.; Yang, H. K.; Lee, C. Amplification of transglutaminase 2 enhances tumor-promoting inflammation in gastric cancers. Exp. Mol. Med. 2020, 52, 854–864.

(20) Pinkas, D. M.; Strop, P.; Brunger, A. T.; Khosla, C. Transglutaminase 2 undergoes a large conformational change upon activation. PLoS Biol. 2007, 5, e327.

(21) Andre, W.; Nondier, I.; Valensi, M.; Guillonneau, F.; Federici, C.; Hoffner, G.; Dijan, P. Identification of brain substrates of transglutaminase by functional proteomics supports its role in neurodegenerative diseases. Neurobiol. Dis. 2017, 101, 40–58.

(22) Biberoglu, K.; Schopfer, L. M.; Tacal, O.; Lockridge, O. Characteristic fragment ions associated with dansyl cadaverine and biotin cadaverine adducts on glutamine. Anal. Biochem. 2020, 600, No. 113718.

(23) Schopfer, L. M.; Lockridge, O. Signature Ions in MS/MS Spectra for Dansyl-Aminoheoxy-QQV Adducts on Lysine. Molecules 2020, 25, 2659.

(24) Miller, M. L.; Johnson, G. V. Transglutaminase cross-linking of the tau protein. J. Neurochem. 1995, 65, 1760–1770.

(25) Singh, U. S.; Pan, J.; Kao, Y. L.; Joshi, S.; Young, K. L.; Baker, K. M. Tissue transglutaminase mediates activation of RhoA and MAP kinase pathways during retinoic acid-induced neuronal differentiation of SH-SY5Y cells. J. Biol. Chem. 2003, 278, 391–399.

(26) Smethurst, P. A.; Griffin, M. Measurement of tissue transglutaminase activity in a permeabilized cell system: its regulation by Ca2+ and nucleotides. Biochem. J. 1996, 313, 803–808.

(27) Thorne, G. C.; Ballard, K. D.; Gaskell, S. J. Metastable decomposition of peptide [M+H]+ ions via rearrangement involving loss of the C-terminal amino acid residue. J. Am. Soc. Mass Spectrom. 1990, 1, 249–257.

(28) Fusus, L.; Tarcza, E. Formation of N epsilon-(gamma-glutamyl)-lysine isodipeptide in Chinese-hamster ovary cells. Biochem. J. 1989, 263, 843–848.

(29) Pavlyukov, M. S.; Antipova, N. V.; Balashova, M. V.; Shakhparonov, M. I. Detection of Transglutaminase 2 conformational changes in living cell. Biochem. Biophys. Res. Commun. 2012, 421, 773–779.

(30) Yamaguchi, H.; Wang, H.-G. Tissue transglutaminase serves as an inhibitor of apoptosis by cross-linking caspase 3 in thapsigargin-treated cells. Mol. Cell. Biol. 2006, 26, 569–579.

(31) Siegel, M.; Strnad, P.; Watts, R. E.; Choi, K.; Jabri, B.; Omary, M. B.; Khosla, C. Extracellular transglutaminase 2 is catalytically inactive, but is transiently activated upon tissue injury. PLoS One 2008, 3, No. e1861.

(32) Badaru, E.; Yang, W.; Rathbone, D. L.; Costanzi, A.; Thibault, T.; Murdoch, C. E.; El Alaoui, S.; Bartkeviciute, M.; Griffin, M. Development of Potent and Selective Tissue Transglutaminase Inhibitors: Their Effect on TG2 Function and Application in Pathological Conditions. Chem. Biol. 2015, 22, 1347–1361.

(33) Kiraly, R.; Demeny, M.; Fusus, L. Protein transamidation by transglutaminase 2 in cells: a disputed Ca2+-dependent action of a multifunctional protein. FEBS J. 2011, 278, 4717–4739.

(34) Sugimura, Y.; Hosono, M.; Kitamura, M.; Tsuda, T.; Yamanishi, K.; Maki, M.; Hitomi, K. Identification of preferred substrate sequences for transglutaminase 1–development of a novel peptide that can efficiently detect cross-linking enzyme activity in the skin. FEBS J. 2008, 275, 5667–5677.

(35) Hitomi, K.; Kitamura, M.; Sugimura, Y. Preferred substrate sequences for transglutaminase 2: screening using a phage-displayed peptide library. Amino Acids 2009, 36, 619–624.

(36) Sugimura, Y.; Hosono, M.; Wada, F.; Yoshimura, T.; Maki, M.; Hitomi, K. Screening for the preferred substrate sequence of transglutaminase using a phage-displayed peptide library: identification of peptide substrates for TGase 2 and Factor XIIIa. J. Biol. Chem. 2006, 281, 17699–17706.

(37) Keresztessy, Z.; Csoon, E.; Harsfalvi, J.; Csomos, K.; Gray, J.; Lightowler, R. N.; Lakey, J. H.; Balajthy, Z.; Fusus, L. Phage display selection of efficient glutamine-donor substrate peptides for transglutaminase 2. Protein Sci. 2006, 15, 2466–2480.

(38) Bui- Nguyen, T. M.; Dennis, W. E.; Jackson, D. A.; Stallings, J. D.; Lewis, J. A. Detection of dichlorvos adducts in a hepatocyte cell line. J. Proteome Res. 2013, 14, 3583–3595.

(39) Ogawa, H.; Goldsmith, L. A. Human epidermal transglutaminase. Preparation and properties. J. Biol. Chem. 1976, 251, 7281–7288.