

Supplemental material for article:

Transglutaminase 6: A protein associated with central nervous system development and motor function

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Materials and Methods

Cloning of human TG6 by anchored PCR

For cloning of TG6, poly(A)⁺RNA was prepared from about 10⁶ H69 cells by oligo(dT)-cellulose column chromatography using the Micro-Fast Track Kit (Invitrogen) and recovered in 20µl 10mM Tris/HCl, pH 7.5. The poly(A)⁺RNA (5.0µl) was reverse transcribed into DNA in a total volume of 20µl using the cDNA Cycle Kit (Invitrogen) with 1.0µl oligo(dT) primer (0.2µg/µl). Overlapping fragments of TG6 were amplified by PCR using oligonucleotides 5'-ATCAGAGTCACCAAGGTGGAC (fP1), 5'-AGAAACACATCGTCCTCTGCACACC (rP1) (0.43Kb), 5'-CAGGCTTTCCTCTCACCGCAAACAC, 5'-CGTACTTGACTGGCTTGTACCTGCC (0.46Kb), 5'-TCTACGTCACCAGGGTCATCAGTGC, 5'-GCCTGTTACCGCCTTGCTGT (0.74Kb), 5'-CATCACTGACCTCTACAAGTATCC, 5'-ACGGCGTGGGATTCATGCAGG (0.35Kb), 5'-CATCCTCTATACCCGCAAGCC (fP2), and 5'-AGGTTGAGGCAGGATTAAGTGGCCTC (0.6Kb). PCRs were carried out with 1.25 units of *AmpliTaq* Gold DNA polymerase (Applied Biosystems) and 2.0µl cDNA in a total of 50µl of supplied reaction buffer supplemented with 2mM MgCl₂, 0.2mM dNTPs and 25pmol of the appropriate gene-specific primers. A total of 40 PCR cycles were made, with an elevated annealing temperature of 65°C for the initial 5 cycles and an annealing temperature of 60°C for the remaining cycles. The 5'-end of the cDNA was isolated by 5'-RACE PCR essentially as described previously (Grenard et al. 2001). After polyC-tailing the cDNA, the PCR reaction was anchored by performing a total of 5 cycles of one-sided PCR at lower annealing temperature (37°C) with the abridged anchor primer (Life Technologies, Inc.) only. Following transfer of 25% of this reaction at 94°C to a new tube containing abridged anchor primer and 5'-GATGTCTGGAACACAGCTTTGG the first round of amplification was carried out for a total of 40 cycles with an annealing temperature of 55°C. Nested PCR was done with the universal amplification primer (Life Technologies, Inc.) and TG6-specific oligonucleotides 5'-TCACAGTCCAGGGCTCTGCTCAG (rP2) or 5'-GCGTGAGGCTGAACGACTGG as described above (annealing at 60°C) using 1.0µl from the first round PCR. The PCR-products were either directly sequenced or, when desired, cloned by taking advantage of the 3' A-overhangs generated by Taq DNA polymerase using the Original TA-Cloning Kit (Invitrogen). Cycle sequencing was performed by the dideoxy chain termination method using the dRhodamine Terminator Cycle Sequencing Ready reaction kit (Applied Biosystems) and an ABI 310 automated sequencer.

Primer Extension Analysis

50pmol oligonucleotide 5'-Cy5-ACCGTCAGGACAGTGTGGC (Sigma Genosys) was hybridized to 0.9µg of H69 cell poly(A)⁺RNA and reverse transcription performed with 200units of Superscript II RNase H⁻ reverse transcriptase (Invitrogen) in a total of 20µl for 60min at 42°C according to the manufacturer's instructions. The enzyme was heat inactivated and primer extension products extracted with phenol chloroform, precipitated with ethanol, and then analyzed separately or after mixing with 5fmol of ALFexpressTM Sizer 50-500 (Amersham Biosciences) on a 6% denaturing polyacrylamide gel in the Open GeneTM System Long Read Tower (Visible Genetics). Fragment analysis was carried out with the Gene ObjectsTM software package. The transcription initiation site was subsequently verified by PCR using oligonucleotides immediately upstream (5'-AGGGGAGAGGGAGCCACAGA) or downstream (5'-GCAACCATGTACTTCGG) of this site together with rP2.

Northern blotting

5µg of poly(A)⁺RNA from H69 cells was separated in a 1.2% agarose gel containing formaldehyde and transferred to a Nytran nylon membrane (Schleicher & Schuell). The gel was calibrated using the 0.24-9.5 kb RNA ladder (Life Technologies). A ~730bp DNA fragment encoding the 3'-end of TG6 was amplified by PCR using oligonucleotides 5'-AGGGCTGGGGGTCGCTGTC and 5'-AATCCACCCCTCTCAGTCCCT and cloned into the pCRII vector using TA cloning. A digoxigenin-UTP (DIG)-labeled single-strand RNA probe was prepared using the DIG RNA Labeling Kit (Roche). The membrane was hybridized with 100ng/ml DIG-labeled RNA probe at 42°C overnight and washed with a final stringency of 0.1 x SSC, 0.1% SDS at 65°C for 30min. DIG was detected with alkaline phosphatase-conjugated anti-DIG antibodies and chemiluminescent detection with CDP star (DIG Nucleic Acid Detection kit, Roche). The membrane was exposed to BioMax MR film (Kodak, Rochester, NY) for 25min.

Cloning of mouse TG6 and PCR amplification of TGs from mouse brain cDNA

Mouse brain (BALB/c) was dissected, rinsed in PBS and immediately frozen on dry ice. The frozen brain was homogenised in 1ml of Tri Reagent (Sigma) using a teflon pestle and total RNA was prepared by chloroform extraction and isopropanol precipitation following the manufacturer's instructions. Where indicated, poly(A)⁺RNA was prepared using the Micro-Fast Track Kit (Invitrogen). cDNA was synthesized from 2µg total RNA or 500ng poly(A)⁺RNA by reverse transcription using 200 units of SuperScript II RNase H⁻ Reverse Transcriptase (Invitrogen) and 0.5µM oligo(dT)₁₅ primer in a total volume of 20µl. Overlapping fragments of TG6 were amplified by PCR using oligonucleotides fp1 and 5'-ATCCTGGTGACATAGACTGGG, 5'-GAGGACGATGTGTTTCTGGAC and 5'-ATCCGCCTCCTCCTCCCC, 5'-CAGCCTCAGTCACCGCCATCCGC and 5'-TTCTTTCTAGTGGCCCCAGC, fp2 and 5'-CCACCATCAGCGCAGAGTCC, 5'-CAGCAGTGGTAGGAGTGACAG and 5'-GAGGGTGAAGGGTTACTGAGTC. The oligo(dT)-*Not I* primer (Invitrogen) was used as the 3' anchoring primer together with 5'-ACAGTTCCTCCAGACATCAAGG for the 3'-end of the cDNA. For 5'-RACE PCR, the first round of amplification was carried out with gene specific oligonucleotide 5'-TGTCTGTTCTCTTTTGCTGC and the second round with 5'-AGGGTGAGACTGAATAACTGG or 5'-GTAATCTTGGGTGTGGTGGG as outlined above.

TG gene product-specific primers were: TG1, 5'-CTCGGACTCTGTGACCATGC and 5'-CAACTGCTGCTCCCAGTAACG; TG2, 5'-CGAATCCTCTACGAGAAGTACAGC and 5'-CAGTTTGCGGTTTTGCTTGG; TG3, 5'-GCAGCCTCAAGATTGATGTGC and 5'-AGACATCAATGGGCAGCATGG; TG4, 5'-CCCTGTGTTTCCTGTACTCTGC and 5'-ACAATGAATTTCTGGGGTCC; TG5, 5'-AGCAGCAGAGAGTTCTCATTGG and 5'-CCTTGATGTCTTAAACCTATTGCTC; TG6, 5'-GGCAGTGACTCTCGTGTGGAC and 5'-TCACGGTGTGGGATTCACGC; TG7, 5'-GGGAGTGGCCTCATCAATGGG and 5'-CCTTGACCTCACTGCTGCTGA; factor XIIIa, 5'-GACCCAAGAGAAAGGTGTTCCG and 5'-GTAGGTCGTCTTTGAATCTGAAG; and band 4.2 protein 5'-CAGGAGGAGTAAGGGGAATTGG and 5'-TGCAGGCTACTGGAATCCACAG. PCRs were carried out with 1.25 units of *AmpliTaq* Gold DNA polymerase (Applied Biosystems) and 1µl mouse brain cDNA in a total of 50µl of 15mM Tris/HCl, pH 8.0, 50mM KCl supplemented with 2mM MgCl₂, 0.2mM dNTPs and 0.5µM of the selected TG specific primers. 35 cycles were carried out in an ABI GeneAmp 9700 thermal cycler, each cycle consisting of denaturation at 94°C for 45s, annealing at 60°C for 1 min and extension at 72°C for 1.5 min. Products were analysed on 1% agarose gels, extracted using the QIAquick Gel Extraction kit (Qiagen), and sequenced by the Sanger dideoxy method using the Dual CyDye Terminator sequencing kit (Amersham Biosciences) and the Open GeneTM System Long Read Tower (Visible Genetics). Alternative splicing of exon 3 or exon 3/4 was tested using oligonucleotides 5'-ATTACCCTTGCTGTGAAGTGG and 5'-ATCCTGGTGACATAGACTGGG and exon 12 using oligonucleotides 5'-CAAGAAGATCCTGTTGGCTGC and 5'-GAGGGTGAAGGGTTACTGAGTC.

ELISA-style transamidation assay

TG activity was determined by measurement of the incorporation of biotin-x-cadaverine (Molecular Probes) into *N,N*-dimethylcasein. Briefly, 96-well plates (Nunc) were coated with 100µg/ml *N,N*-dimethylcasein and incubated at 37°C for 1h before blocking with 3% casein (30 min at room temperature). The reaction mixture contained 0.4mM biotin-x-cadaverine, 10mM DTT, 1mM CaCl₂ or 10mM EGTA and the indicated concentration of enzyme. Recombinant TG2 and TG6 was produced in *E.coli* as described (Hadjivassiliou et al., 2008). The reaction was incubated for the indicated time at 37°C and stopped by adding 200mM EGTA. Amine incorporation was detected by incubation with horse radish peroxidase (HRP)-conjugated avidin (BioRad) for 1h at room temperature and bound HRP visualized with ABTS [2,2-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] as a substrate, and the absorbance was measured at 405nm.

Real-time fluorescence assay for determination of TG isopeptidase activity

TG activity was quantified by measuring changes in fluorescence intensity over time as a result of cleavage of Abz-APE(γ-cad-Dnp)QEA (A102; Zedira, Darmstadt, Germany) in black optical bottom 96 well plates (165305, Nunc). The reaction was carried out in 0.1ml of pre-warmed (37°C) 50mM Tris/HCl, pH 7.4, 100mM NaCl (for TG2) or 300mM NaCl (for TG6), 50µM A102, 5mM DTT, 56mM glycine methylester and 20µg/ml TG6 (Zedira) or TG2. Nucleotides (GTP, GTPγS, GDP, GMP and ATP (Sigma)) were added at the desired concentration either in the absence or presence of MgCl₂ (0.2-20mM), and reaction mixtures were incubated at 37°C for 15 min before enzyme activation by injection of 2mM CaCl₂ (or H₂O as a control). Reaction kinetics were captured over a 1 h period at 37°C using a FLUOstar Optima plate reader (BMG LABTECH) (excitation filter 320 nm, emission filter 440 nm, gain = 2450, 40 s measurement intervals).

In situ hybridization

A 325bp fragment corresponding to the 3' end of mouse TG6 was generated by PCR using oligonucleotides 5'-CATCCTCTATACCCGCAAGCC and 5'-CCACCATCAGCGCAGAGTCC. The fragment was cloned into the pCRII vector using TA cloning (Invitrogen). For *in vitro* transcription, the cDNA fragment with flanking RNA polymerase promoters was excised by restriction with *Pvu*II and *Afl*III and isolated using the QIAquick Gel extraction kit (Qiagen). Digoxigenin-UTP (DIG)-labeled, single-strand antisense and sense RNA probes were prepared using the DIG RNA Labeling Kit (Roche) with 1µg purified DNA fragment and 40 units of either RNA Polymerase SP6 or T7 in 20µl transcription buffer supplemented with 0.1mM ATP, 0.1mM CTP, 0.1mM GTP, 65µM UTP, and 35µM DIG-11-UTP following the manufacturer's instructions. DNA template was degraded by incubation with 20 units RNase-free DNase I (Roche) for 15 min at 37°C, the reaction terminated by addition of 0.2M EDTA (pH 8.0) to a final concentration of 20mM, and after supplementation with 0.4M LiCl, the labeled RNA collected by ethanol precipitation. To determine the yield of labeled RNA, the RNA was compared to a dilution series of a DIG-labeled control RNA spotted onto a Nytran nylon membrane (Schleicher and Schuell) and visualized using the DIG Nucleic Acid Detection kit (Roche). Similar DIG-labeled single stranded sense and antisense RNA probes were generated using a 387bp DNA fragment of TG5 generated with oligonucleotides 5'-AGCCCCCTGGTTCCATTCTGG and 5'-GGCTTTGTGATGGGGTTTGAGGACG.

Newborn BALB/c mice and mouse embryos at gestation days 9, 11, 13 and 16 were fixed in 4% paraformaldehyde in PBS, pH 7.4, at 4°C overnight. To improve penetration of fixative into the newborn mouse, an excision was made along the abdomen with a scalpel. For adult mice, brain was dissected. The mice were transferred to 0.5% paraformaldehyde in PBS containing 0.42M EDTA

for 4 days for demineralisation of skeletal tissues, washed in PBS, processed through a graded ethanol series and paraffin embedded. Sagittal sections of 5µm thickness were cut, transferred onto gelatin coated glass slides and dried overnight at 50°C. Following deparaffinisation, tissue sections were washed with DEPC treated H₂O, treated with proteinase K (Sigma; 4µg/ml in 100mM Tris/HCl, pH 8, 50mM EDTA) at 4°C for 15 min before being acetylated with 0.25% (v/v) acetic anhydride in 0.1M triethanolamine, pH 8.0 for 10 min. Sections were prehybridised for 15 min at 37°C in 5xSSC, followed by hybridisation overnight at 42°C with 20ng/µl DIG-labeled probe in 30µl hybridisation solution consisting of 50% formamide, 10% dextran sulfate, 5xSSC and 300µg/ml herring sperm DNA (Sigma) in DEPC treated H₂O (Frame-Seal incubation chambers [MJ Research, Inc.] were used to eliminate evaporation of reagents during hybridisation). Slides were subsequently washed in 2x SSC for 30 min at room temperature, twice in 2x SSC for 20 min at 37°C, and once in 1x SSC for 20 min at 37°C. Hybridised probe was subsequently visualised using the DIG Nucleic Acid Detection kit (Roche) according to the manufacturer's instructions by incubation with alkaline phosphatase-labeled anti-DIG antibody (diluted 1:500) and subsequent development with NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate) substrate solution for 12 h.

Immunolabeling of cells and FACS analysis

Cells were permeabilized in PBS containing 0.5% saponin for 20 min on ice. After three washes in PBS containing 0.1% saponin, non-specific binding was blocked in TBS containing 1% BSA and 3µg/ml rabbit anti-mouse IgG (Dako) and then cells incubated with monoclonal antibodies against glial fibrillary acidic protein (G-A-5, Sigma; 15µg/ml), β-tubulin isoform III (SDL.3D10, Sigma; 20µg/ml), or RIP (Chemicon, 1:1000 diluted) and goat anti-TG6 antibodies (20µg/ml) in TBS/BSA overnight at 4°C. Primary antibody binding was detected by incubation with FITC-conjugated rabbit anti-mouse (MP Biomedicals) and R-phycoerythrin rabbit anti-goat (Sigma) (for FACS) or rhodamine-conjugated rabbit anti-goat (ICN Biomedicals, Inc.) (for immunocytochemistry) secondary antibodies for 1 h at room temperature. Analysis was performed immediately after labeling using a FACScalibur flow cytometer (Becton Dickinson) equipped with an argon laser emission wavelength of 488nm. FITC and PE signals were identified using 530nm and 585nm band pass filters, respectively. The analysis was performed using Cell Quest software (Becton Dickinson). Ten thousand events were acquired for each sample. Background level of fluorescence was determined from controls with non-specific IgG (ChromPure goat/mouse IgG, Jackson ImmunoResearch Labs, Inc.) replacing the primary antibodies.

Figure Legends

Fig. 1

Nucleotide and deduced amino acid sequences of full-length human and mouse TG6. Triangles indicate exon boundaries. The translation initiation and termination codons as well as the polyadenylation signal (AATAAA) are underlined.

Fig. 2

Primer extension analysis of poly(A⁺) RNA isolated from H69 cells. The size of the reverse transcribed DNA fragments obtained with a fluorescently labeled reverse primer was determined on a polyacrylamide sequencing gel using a fluorescently-labeled 50-500 nucleotide calibration ladder and an automated DNA sequencer. Extension products were run mixed with the DNA ladder as well as separately (not shown) with the same result. The peak representing the major transcription start site is indicated by an arrow together with its size. The genomic sequence of the sense strand between 401 and 450 nucleotides is given on the top with the transcription initiation site indicated as +1. The location of the transcription initiation site was subsequently verified by PCR using sense oligonucleotides immediately upstream or downstream of this site whereby only the latter yielded a product.

Fig. 3

Nucleotide and deduced amino acid sequence for human TG6-S which lacks exon 12. Only the sequence spanning exon 11 and exon 13 is shown. The stop codon is underlined and the triangle indicates the exon boundary.

Fig. 4

Analysis of TG6 expressing cells isolated from cerebral cortex of newborn mice for cell type specific markers by FACS. TG6 positive cells formed two clusters R1 and R2 (top right panel). Below, the TG6 positive cells gated in area R1 (left panels) and R2 (right panels) are plotted in relation to the expression of the markers for distinct cell populations: β -tubulin III for neurons, glial fibrillary acidic protein (GFAP) for astrocytes and RIP for oligodendrocytes. The top left panel corresponds to the analysis of the physical parameters of the global cell population, with distinct cell populations identified by markers indicated. TG6 positive cells gated in area R1 and R2 are highlighted in green and brown, respectively. Controls with non-specific Ig of relevant species are given in bottom panels.