Tissue transglutaminase (tTG) exhibits a magnesium-dependent GTP/ATPase activity that is involved in the regulation of the cell cycle and cell receptor signaling. The portion of the molecule involved in GTP/ATP hydrolysis is unknown. We expressed and purified a series of C-terminal truncation mutants of human tTG as glutathione S-transferase fusion proteins (ΔS538, ΔE447, ΔP345, ΔC290, ΔV228, and ΔF185) to determine the effect on GTP/ATPase activity. The truncation of the C terminus did not change significantly the apparent Kære value for either GTP or ATP. In contrast, the Kære value for GTP was increased by 4.6- and 3-fold for the ΔS538 and ΔE447 mutants, respectively. The ΔP345 mutant had the highest hydrolysis activity with a 34-fold increase. The hydrolysis activity then declined to 8.1-, 8.7-, and 1.9-fold for the ΔC290, ΔV228, and ΔF185 mutants, respectively. The Kære for ATP changed in parallel with the GTPase results. Thin layer chromatography analysis of the hydrolysis reaction products revealed that ATP was rapidly converted to ADP followed by a much slower conversion of ADP to AMP when incubated with wild type tTG or the ΔP345 mutant. There was a substantial decrease in the calcium-dependent TGase activity when the last 149 amino acid residues were deleted from the C terminus. Less than 5% of the TGase activity was detected for the ΔS538 and ΔE447 mutants. In conclusion, we have located the ATP and GTP hydrolytic domain to amino acid residues 1-185. The C terminus functions to inhibit the expression of endogenous GTP/ATPase activity of tTG, and the potential role of the C terminus in modulating this activity is discussed.

Tissue transglutaminase (tTG) is a unique member of the transglutaminase gene family in that it exhibits two distinct enzyme activities (1–3). The calcium-dependent transglutaminase activity (TGase) catalyzes the covalent modification of proteins by the formation of γ-glutamyl-ε-lysine bonds between proteins or polyamines (1, 2). The TGase activity is considered to be an important intracellular and extracellular reaction during apoptosis (4, 5), bone ossification (6), tissue repair (7), and tumor growth (8). TGase activity requires a calcium binding site and active site cysteine to form a thioester bond with the glutamine substrate (1, 2). The active site of human tTG is located at Cys-277, and the putative calcium binding site is located between amino acids 446 and 453 based on sequence homology to the calcium binding site in the factor XIII A chains (9).

The tTG will selectively modify a group of protein-bound glutamine residues that exist in proteins found in the extracellular matrix (ECM) including vitronectin, fibronectin, osteonectin, and nidogen (1, 2). When tTG is released into plasma or ECM it binds to fibronectin and retains TGase activity (1, 2). Fibronectin binding functions to localize tTG to sites of fibronectin expression and deposition and limits the availability of the enzyme for cross-linking other substrates. The fibronectin binding site is located in the N-terminal seven amino acid residues (10).

The tTG binds GTP and ATP inducing a conformational change that causes a reduction in the affinity for calcium and in TGase activity (11, 12). The binding of ATP and/or GTP to intracellular tTG could play a major role in suppressing TGase activity and preventing intracellular protein cross-linking reactions. In addition, a magnesium-dependent GTP hydrolysis activity (GTPase) was discovered to reside in the molecule and does not require the active site cysteine (13). The over-expression of a tTG mutant with the active site Cys-277 mutated to alanine only expressed GTPase activity and caused cell cycle arrest at the S to G/M interphase (14). The GTPase activity of the tTG was also reported to function in cell receptor signaling by the α1-adrenoreceptor (15). These recent studies emphasize the importance of understanding the molecular basis for regulating the TGase and GTPase activity.

Takeuchi et al. (16) reported three potential nucleotide binding sites located at amino acid residues 46–69, 345–367, and 520–544 of guinea pig tTG based on the ability of peptides to bind either GTP or ATP directly (16). A 36-kDa N-terminal fragment was purified from rabbit liver nuclei that could bind GTP, suggesting that the N terminus of tTG plays an important role in nucleotide binding (17). 63- and 37-kDa N-terminal fragments of tTG were detected in a human erythroleukemia cell line, raising the possibility that an alternative splicing of mRNA could produce a protein that plays a role in leukemia cell proliferation (18, 19). The C-terminal eight amino acid residues of tTG were recently reported to associate with the recognition and stimulation of phospholipase C (20).

The purpose of this study was to perform C-terminal deletion analysis of the recombinant human tTG and determine the effect on GTP/ATP hydrolysis (nucleotide triphosphatase,
NTPase) activity of human tTG. We report that C-terminal deletion loss a cause of TGase activity and a major increase in NTPase activity. All mutants retained the fibronectin binding property. The importance of the C terminus in regulating the NTPase function will be discussed.

MATERIALS AND METHODS

Reagents—Sodium salts of ATP, ADP, AMP, GTP, GDP, and GMP were the highest quality available and were purchased from Sigma. [γ-32P]GTP (or ATP) and [α-32P]ATP (30 Ci/mmol) were purchased from Du Pont NEN. All ATP, ADP, AMP, GTP, GDP, and GMP solutions were prepared in 50 mM Tris acetate, pH 7.0, and stored in aliquots at −80 °C. Restriction enzymes, T4 DNA ligase, Luria-Bertani (LB) medium, and most reagents were obtained from Life Technologies, Inc. All other reagents used in this investigation were purchased from Sigma unless stated otherwise.

Assembly of Human tTG cDNA—Briefly, the full-length human tTG cDNA was assembled by ligating a partial cDNA clone (encoding Leu-197 to Val-491) kindly provided by Gentile et al. (9), a 5′-end cDNA (encoding Met-1 to Leu-196), and a 3′-end cDNA (encoding Phe-492 to Ala-887). The 5′- and 3′-end cDNAs of human tTG were synthesized by reverse transcription of total RNA prepared from microvascular endothelial cells using the superscript II reverse transcriptase (Life Technologies, Inc.) and polymerase chain reaction amplification using Taq polymerase (Life Technologies, Inc.). The assembled full-length cDNA was subcloned into the Neo and HindIII sites of the pEX-MCS expression vector (27) and designated as pl-TG. DNA sequencing analysis demonstrated that there were polymorphisms at amino acid Glu-186 (GAA), Asn-533 (AAC), and Leu-655 (CTG) since they are either present in other transglutaminases or in the alternatively spliced form of tTG, respectively. All deletion mutants were confirmed by sequencing.

In vitro expression and purification of recombinant human tTG demonstrated properties indistinguishable from tTG purified from other sources.

Construction of C-Terminal Deletion Mutants—All mutants were constructed using oligonucleotide-directed mutagenesis according to manufacturer’s instructions (Chameleon In vitro mutagenesis kit, Stratagene). pG-TG was used as the DNA template for mutagenesis (21). The deletion mutants ΔS538, ΔE447, ΔP345, ΔC290, ΔV228, and ΔF185 were constructed by engineering a TAA stop codon at the amino acids 533, 446, 439, 291, 229, and 186 using oligonucleotide 1 (CTTGG AGCCCT TCTTCA AGAGG AGACG GTCGC), 2 (CTCTAC AAATA CCCAT CCTAC AAATA CCCAT CCTAC AAATA CCCAT CCTAC AAATA CCCAT), and 3 (GGTGG GTAGT GGCAT GTAAA ACTGC AACGA CCCTA CC), 4 (GGTGG GTAGT GGCAT GTAAA ACTGC AACGA CCCTA CC), 5 (GGTGG GTAGT GGCAT GTAAA ACTGC AACGA CCCTA CC), and 6 (CCCTA CC), respectively. All deletion mutants were purified by DNA sequencing.

Expression and Purification of Recombinant tTG—The conditions for growing the Escherichia coli and purification of the GST fusion proteins were as described previously (21). All truncation mutants were purified in triplicate. Preliminary studies demonstrated that deviation of the TGase and GTP/ATPase activities measured in this study were less than 5% among the triplicate preparations. Affinity-purified fusion protein migrated in the SDS-polyacrylamide gels as the alternatively spliced form of tTG, i.e. tTG homologue (18). Furthermore, based on mobility on SDS-polyacrylamide gel, fibronectin binding, TGase, and GTP/ATPase activities, the affinity-purified recombinant human tTG demonstrated properties indistinguishable from tTG purified from other sources.

Denaturation and Renaturation of GST-tTG and GST-P345—The protocol for denaturation and renaturation was adapted from the procedures established for plasma transglutaminase (25). GST-tTG and GST-P345 (100 µg/ml) were denatured in a solution containing 20 mM Tris-Cl, pH 8.0, 100 mM NaCl, 1 mM dithiothreitol/EDTA, and 6 mM guanidinium HCl at room temperature for 1 h. Renaturation was performed by dialyzing the denatured GST-tTG and GST-P345 either in a solution containing 20 mM Tris-Cl, pH 8.0, 100 mM NaCl, 1 mM dithiothreitol/EDTA (buffer A) at 4 °C for 16 h or in buffer A containing 1 mM urea for 8 h and then in buffer A for additional 8 h. After dialysis, the samples were centrifuged at top speed in Eppendorf’s microcentrifuge (model 5415C) for 30 min to remove the insoluble proteins. Control experiments were also performed by dialyzing the same concentration of GST-tTG and GST-P345 without a denaturation step to examine the stability of the proteins under the same conditions. The proteins were analyzed by 9% SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue. The results demonstrated that there were no degradation of proteins. The TGase and GTP/ATPase activities were also measured according to the procedures as described above.

RESULTS AND DISCUSSION

We investigated the role of the C terminus in regulating the GTP/ATPase activity of human tTG by expressing and purifying C-terminal deletion mutants as GST fusion proteins. We initially constructed mutants ΔS538, ΔE447, and ΔP345 (Fig. 1A). The ΔS538 mutant is similar in size to a tTG homologue expressed in human erythroblastic cells and was recently reported to have increased GTPase activity (26). The ΔE447 mutant lacks the putative calcium binding domain (amino acids 446–453). The ΔP345 was designed to remove additional charged residues that could play a role in GTP and ATP binding (16). Based on the sequence alignment with the structural domains of factor XIII A chain (27), the truncation site Ser-538 was located in the barrel 1 region, and Glu-447 and Pro-345 were located in the catalytic core region (Fig. 1A). Since the GTP/ATP hydrolysis activity was increased by 34-fold in the ΔP345 mutant (Fig. 1, C and D, see below), additional C-terminal truncation mutants were constructed. The ΔC290, ΔE447, and ΔF185 mutants were constructed by engineered stop codons for exon 5, 4, and 3, respectively. These truncation sites were located in the catalytic core region when aligned with factor XIII A chain (Fig. 1A). All mutants were expressed and purified containing equivalent amounts of GST protein was used as blank value to subtract from the sum of the kinetic constant, K_m, K_cat, and V_max, the concentrations of GTP and ATP were varied from 4 to 20 µM. Kinetic analysis of the data was performed by the Eadie-Hofstee method.

Quantitation of ATP Hydrolysis and Analysis of Reaction Products by Thin Layer Chromatography—The ATP hydrolysis was performed as described in an earlier section except [α-32P]ATP was used. After the reaction, 45 µl of the reaction mixture was mixed with 4.5 µl of 50% perchloric acid and 5.25 µl of 46.4% KOH and incubated at 4 °C for 30 min. After centrifugation at 12,000 rpm for 1 min, 3 µl of the supernatant was applied to a PEI-cellulose TLC plate (EM Science, Gibbstown, NJ) and developed at room temperature in a buffer containing 1 mM LiCl and 0.6 M acetic acid for 2 h. The TLC plate was dried and subjected to autoradiography. The position of ATP, ADP, and AMP was identified by applying unlabeled ATP, ADP, and AMP standards on the same plate.

The intensity of the bands was quantified by a Molecular Dynamics phosphorimage and ImageQuant software.

Fibronectin Binding Assay—ELISA microtiter plates (Costar; Cambridge, MA) were coated overnight at 4°C with 100 µl of 50 µg/ml human fibronectin (Sigma) followed by blocking with 200 µl of 1% BSA in TBS (0.1 M Tris-HCl, 0.15 M NaCl, pH 8.5) at 37 °C for 60 min. GST-tTG or C-terminal truncation mutants (2 × 10^−6 to 20 µg/100 µl in 1% BSA, 0.05% Tween, and TBS) in duplicate was added and incubated for 2 h at 37 °C. After washing three times with TBS plus 0.05% Tween (203B), 100 µl of anti-tTG monoclonal antibody, CUB 7401 (1:1000 dilution in 1% BSA/TBST) was added and incubated at 37 °C for 2 h after washing three times with TBS, goat anti-mouse IgG (H + L) streptavidin-alkaline phosphatase (1:1000 dilution in 1% BSA/TBST) was added and incubated at 37 °C for 60 min. The color was developed using 1 mg/ml of p-nitrophenyl phosphate (Pierce). An endpoint O.D. reading at 405 nm was obtained at 30 min.
as GST-fusion proteins and migrated on SDS-polyacrylamide gel electrophoresis with their expected molecular weights (Fig. 1A). All mutants except ΔC290 were shown to be greater than 90% pure by Coomassie Blue staining after SDS-polyacrylamide gel electrophoresis separation. The ΔC290 was approximately 50% pure and consistently had an E. coli protein copurify with the mutant. In preliminary studies, we established that cleavage of the GST was not necessary to obtain reproducible TGase activity measurements, GTPase/ATPase hydrolysis, and fibronectin binding results. The results obtained with the GST fusion protein were comparable with those obtained from cleaving the GST and purifying the tTG. Therefore, the affinity-purified fusion proteins were used directly in all investigations to facilitate the rapid purification of the mutants by the same method.

We found there was a substantial decrease in the calcium-dependent TGase activity when the 149 amino acid residues were deleted from the C terminus (Fig. 1B). From 1 to 5% of the original TGase activity was detected for the ΔSS538 and ΔE447 mutants. The ΔP345, ΔC290, ΔV228, and ΔF185 mutants had no detectable TGase activity even when assayed at 50-fold higher protein concentration. When the calcium chloride concentration was increased to 100 mM, there was no further increase in the TGase activity for the inactive tTG mutants (Fig. 1C). There was a 34-fold increase in hydrolysis for the ΔP345 mutant and a subsequent reduction in activity to only 8.1-, 8.7-, and 1.9-fold increase in hydrolysis for the ΔC290, ΔV228, and ΔF185 mutants, respectively (Fig. 1D). The change in $K_{cat}$ for ATP increased in parallel with the GTP results, and the highest $K_{cat}$ was detected for the ΔP345 mutant (Fig. 1D). The removal of the C terminus apparently allows the GTP/ATPase catalytic domain to fold in a manner that increases NTPase activity. These data are consistent with published data demonstrating that an N-terminal 36-kDa fragment purified from rabbit liver retained GTP binding properties (17) and that a human tTG homologue found in erythroleukemia cells had higher GTP hydrolysis activity (26). These data localize a GTP/ATPase hydrolytic domain to the N-terminal 185 amino acids of tTG with a predicted Mr of 20,825. It is possible that selective proteolysis of the C terminus of tTG could occur at some intracellular or extracellular compartment to produce a N-terminal fragment with increased NTPase activity. In preliminary studies, we have been able to detect an increase in the GTP/ATP hydrolysis activity of tTG by treating it with several different proteases. This finding demonstrated that the activity obtained with our deletion mutants as GST-fusion proteins and migrated on SDS-polyacrylamide gel electrophoresis with their expected molecular weights (Fig. 1A). All mutants except ΔC290 were shown to be greater than 90% pure by Coomassie Blue staining after SDS-polyacrylamide gel electrophoresis separation. The ΔC290 was approximately 50% pure and consistently had an E. coli protein copurify with the mutant. In preliminary studies, we established that cleavage of the GST was not necessary to obtain reproducible TGase activity measurements, GTPase/ATPase hydrolysis, and fibronectin binding results. The results obtained with the GST fusion protein were comparable with those obtained from cleaving the GST and purifying the tTG. Therefore, the affinity-purified fusion proteins were used directly in all investigations to facilitate the rapid purification of the mutants by the same method.

We found there was a substantial decrease in the calcium-dependent TGase activity when the 149 amino acid residues were deleted from the C terminus (Fig. 1B). From 1 to 5% of the original TGase activity was detected for the ΔSS538 and ΔE447 mutants. The ΔP345, ΔC290, ΔV228, and ΔF185 mutants had no detectable TGase activity even when assayed at 50-fold higher protein concentration. When the calcium chloride concentration was increased to 100 mM, there was no further increase in the TGase activity for the inactive tTG mutants. All of the truncation mutants displayed specific binding to the fibronectin-coated plate, demonstrating that the N terminus was exposed and could bind to fibronectin.

We found that 1 mM MgCl₂ was the optimal concentration required for hydrolysis of both GTP and ATP for all the C-terminal truncation mutants, and we used this concentration to characterize all the mutants. The apparent $K_{cat}$ value for GTP varied from 110 to 181.9 μM and from 30.9 to 43 μM for ATP. These relatively minor changes demonstrate all mutants retained comparable affinity for ATP and GTP. In contrast, the $K_{cat}$ for GTP was increased by 4.6- and 3-fold for the ΔSS538 and ΔE447 mutants (Fig. 1C). There was a 34-fold increase in hydrolysis for the ΔP345 mutant and a subsequent reduction in activity to only 8.1-, 8.7-, and 1.9-fold increase in hydrolysis for the ΔC290, ΔV228, and ΔF185 mutants, respectively (Fig. 1D). The change in $K_{cat}$ for ATP increased in parallel with the GTP results, and the highest $K_{cat}$ was detected for the ΔP345 mutant (Fig. 1D). The removal of the C terminus apparently allows the GTP/ATPase catalytic domain to fold in a manner that increases NTPase activity. These data are consistent with published data demonstrating that an N-terminal 36-kDa fragment purified from rabbit liver retained GTP binding properties (17) and that a human tTG homologue found in erythroleukemia cells had higher GTP hydrolysis activity (26). These data localize a GTP/ATPase hydrolytic domain to the N-terminal 185 amino acids of tTG with a predicted Mr of 20,825. It is possible that selective proteolysis of the C terminus of tTG could occur at some intracellular or extracellular compartment to produce a N-terminal fragment with increased NTPase activity. In preliminary studies, we have been able to detect an increase in the GTP/ATP hydrolysis activity of tTG by treating it with several different proteases. This finding demonstrated that the activity obtained with our deletion mutants

![Fig. 1. A, schematic diagram of factor XIII A chain, wild type tTG, and its C-terminal truncation mutants. The factor XIII A chain structure domains are illustrated to show the relationship with wild type tTG and its C-terminal truncation mutants. The wild type (WT) tTG and its carboxyl-terminal truncation mutants ΔSS538, ΔE447, ΔP345, ΔC290, ΔV228, and ΔF185 were expressed in E. coli as GST fusion protein. The calculated molecular weights of each protein are also shown. B, TGase activity. The transglutaminase activity of each mutant was determined by a 5-biotin (amido)pentylamine incorporation assay as described under "Materials and Methods." The results represent the average of two triplicate experiments and are expressed as a percentage relative to wild type tTG. C and D, $K_{cat}$ for GTP and ATP. The $K_{cat}$ values for GTP and ATP were determined as described under "Materials and Methods." Results were the average of two duplicate experiments and are presented as fold increase relative to wild type tTG. The $K_{cat}$ for GTP and ATP of wild type tTG were 0.06 and 0.08/min, respectively.](image-url)
was not an artifact of expressing C-terminal deletion mutants.\(^2\) Further studies are in progress to test the hypothesis that proteases activate the GTP/ATPase activity.

To investigate whether protein folding is required for GTP/ATPase activity, we selected full-length tTG and \(\Delta P345\) mutant for this experiment. Since the conditions for protein renaturation vary between different proteins, we adapted the conditions that have been established for a homologous protein, plasma transglutaminase (i.e. coagulation factor XIII A chains) (25). The results demonstrated that 90–95% of the protein remained soluble after renaturation. However, only 8–13% and 15% of the original GTPase activity was detected for the full-length tTG and \(\Delta P345\) mutant, respectively. There was no detectable TGase activity after renaturation of either the full-length tTG or the \(\Delta P345\) mutant. These results indicate that proper protein folding is required for displaying the GTP/ATPase and TGase activities. Furthermore, TGase activity is more sensitive to denaturation than the hydrolysis activity of the tTG.

Although other investigators reported that tTG had ATPase activity (28), none of the earlier investigations determined the nature of the reaction products generated by incubating ATP with tTG. The reaction products of ATP hydrolysis for both full-length tTG and the \(\Delta P345\) mutant were studied by TLC as described under “Materials and Methods.” Degradation of ATP occurred in two consecutive steps; there was very rapid formation of ADP followed by a much slower conversion of ADP to AMP. More than 50% of the ATP was converted to ADP during the first 10 min, and 85% was converted to ADP after 30 min of incubation. There was a substantial lag phase for the formation of AMP during the first 40 min of incubation, and less than 5% of the ADP was converted to AMP after 60 min of incubation (results not shown). Control experiments performed with either no enzyme or only the GST protein did not produce any ADP or AMP. These results indicate that the \(\Delta P345\) mutant hydrolyzes ATP to ADP at a much faster rate than the conversion of ADP to AMP. We found a similar pattern of hydrolysis for wild type tTG with more than 90% of the ATP converted to ADP, and <5% of ADP converted to AMP after 60 min of incubation. The inability of tTG to convert ADP to AMP suggests that the ADP could play a role in modulating the intracellular activity of the enzyme. We recently found that ADP can bind and inhibit the ATPase activity of the tTG.\(^2\) Therefore, once ADP is formed it would prevent ATP hydrolysis. In addition, ATP-tTG is inactive while ADP-tTG retains calcium-dependent TGase function.\(^2\)

The majority of tTG is found in the cytoplasm of the cell while 4–20% is associated with the particulate fraction (29). The recent finding that the full-length and the 36-kDa cleaved form of tTG associates with the nuclear pore of rat liver nuclei suggests that the tTG could play a role in modifying the structure of the nuclear pore or in trafficking of molecules through the pore (17). Specific processing by proteases in the nuclei could regulate the TGase and NTPase activity in this cellular compartment and warrants further analysis.

The ECM surrounding endothelial cells and fibroblasts contain tTG (1, 2) and are exposed to ATP and other NTPs at sites of cell and tissue injury. The tTG could play a role in converting ATP to ADP and altering the response of cells to these vasoactive molecules (30). The tTG in the ECM could be cleaved at the C terminus by a protease released in response to tissue injury, and the cleaved form of tTG could modify local nucleotide concentrations. Cleaved forms of tTG were detected in human atherosclerotic aortic tissues and human breast cancer tumor tissue.\(^2\) If the proteolysis of tTG leaves the N terminus intact, fragments would remain bound to ECM and be localized to stimulate platelet activation by converting ATP to ADP. In preliminary studies, we have confirmed these findings by stimulating platelet aggregation by incubating platelet-rich plasma with a solution of ATP previously incubated with proteases and recombinant tTG.\(^2\) Since ATP and ADP are potent bioactive molecules in vascular tissues where tTG is expressed at high levels, the regulation of latent NTPase activity could be an important function for this ECM protein.

Recently, the erythrocyte membrane protein band 4.2 (Pallidin) was shown to bind ATP but not GTP, and the ATP binding consensus sequence was located at amino acids 340–352 (31), which corresponds to amino acids 321–329 of human tTG. An ATP binding consensus sequence does not exist in tTG or any of the other published TG sequence. Since the \(\Delta C290, \Delta V228,\) and \(\Delta F185\) mutants retained significant GTP/ATP hydrolysis, amino acids 321–329 are not essential for hydrolysis, and the GTP/ATP binding capacity must reside in the first 185 amino acid residues.

The C terminus also plays an important role in regulating the TGase activity since the activity of \(\Delta S538\) and \(\Delta E447\) mutants were decreased to 1–5% of the original value. The residual TGase activity displayed by the \(\Delta E447\) mutant suggests that additional calcium binding site(s) could substitute for the putative calcium binding domain located between amino acid residues 446 and 453. In addition, Ikura et al. (32) also reported that the anionic amino acid residues (Glu-445 to Glu-452) in this domain were not essential for guinea pig liver TG to display calcium-dependent TGase activity. Further work is needed to localize the calcium binding site(s) in tTG.

In conclusion, we have localized the ATP and GTP hydrolytic domain to amino acid residues 1–185 in the N terminus of tTG. The predicted structure of this fragment contains \(\beta\)-sandwich domain and part of the catalytic core region when it aligns with the structural domains of factor XIII A chain (27). The C terminus of tTG functions to inhibit the expression of this endogenous GTP/ATPase activity in the intact protein. Studies are in progress to define the role of the NTPase activity of tTG in healthy and injured tissues and define the importance of this reaction in regulating the cell cycle and cell receptor signaling.

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\(^2\) T.-S. Lai and C. S. Greenberg, unpublished findings.
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