Isolation and Characterization of cDNA Clones to Mouse Macrophage and Human Endothelial Cell Tissue Transglutaminases*

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The deduced amino acid sequences for tissue transglutaminases from human endothelial cells and mouse macrophages have been derived from cloned cDNAs. Northern blot analysis of both tissue transglutaminases shows a message size of approximately 3.6–3.7 kilobases. The molecular weights calculated from the deduced amino acid sequences were 77,253 for human endothelial tissue transglutaminase and 76,699 for mouse macrophage tissue transglutaminase. The deduced amino acid sequence for the human endothelial transglutaminase was confirmed by comparison with the amino acid sequence obtained by cyanogen bromide digestion of the human erythrocyte transglutaminase. The amino acid sequences of both human endothelial and mouse macrophage tissue transglutaminases were compared to other transglutaminases. A very high degree of homology was found between human endothelial, mouse macrophage, and guinea pig liver tissue transglutaminase (>80%). Moreover, human endothelial tissue transglutaminase was compared with human Factor XIIIa and a very high degree of homology (75% identity) was found in the active site region.

Transglutaminases (E.C. 2.3.2.13) are calcium-dependent enzymes that catalyze the cross-linking of proteins by promoting the formation of isopeptide bonds between protein-bound glutamine and lysine residues. These enzymes also catalyze the conjugation of polyamines to proteins. Interest in the possible physiological role of protein cross-linking reactions in both normal and pathological processes has led to the identification of transglutaminase activity in many cells and tissues (1). However, the relationship among the enzymes responsible for these activities has remained largely a matter of conjecture. The best characterized transglutaminase, plasma transglutaminase (Factor XIII), (2) has been purified, cloned, and sequenced (3–4). Ikura and colleagues (5) have cloned the transglutaminase from guinea pig liver and have shown that this enzyme (tissue transglutaminase) is distinct from Factor XIII. Recently a second intracellular transglutaminase has been cloned from rabbit epithelial cells and has been shown to be distinct from both Factor XIII and tissue transglutaminase (6). We have been interested in the transglutaminase activity regulated by retinoids (7). Retinoic acid has been shown to induce high levels of transglutaminase activity in myeloid (8, 9) and endothelial cells (10). To extend our knowledge of the relationship among the cellular transglutaminases, we have isolated cDNA's encoding tissue transglutaminase in cells with high constitutive expression (human umbilical vein endothelial cells) and in cells showing a marked induction after stimulation by retinoids (mouse macrophages). We have then compared the deduced amino acid sequences of these enzymes with guinea pig liver tissue transglutaminase and transglutaminases from other species. High homology was found when we compared the enzymes from human endothelial and mouse macrophage cells with guinea pig liver tissue transglutaminase. A lower degree of homology was found when we compared human endothelial and mouse macrophage amino acid sequences (32 and 36%, respectively) with rabbit tracheal epithelial cell transglutaminase.

EXPERIMENTAL PROCEDURES AND RESULTS

Cloning of Tissue Transglutaminases—We have been interested in the factors that regulate the expression of transglutaminases in cells and tissues. In order to pursue this question we have cloned tissue transglutaminase from mouse and human cells. In the case of the mouse tissue transglutaminase, screening of mouse macrophage and heart libraries resulted in the isolation of several overlapping clones. One of them (mouse clone TGHZ3) included 29 nucleotides of 5'-untranslated sequence and 1775 nucleotides of coding sequence. Screening of a human endothelial cell cDNA library, on the other hand, resulted in the isolation of a cDNA clone that included the entire 5'-untranslated sequence (as determined by primer extension analysis), the coding domain, and 1058 nucleotides of 3'-untranslated sequence. This clone lacked a consensus polyadenylation sequence and is slightly shorter than the 3,6 kilobases full-length transcript (as determined by Northern blot analysis of human endothelial cell RNA) suggesting that it lacks approximately 300 base pairs of 3'-untranslated sequence.

We confirmed the identity of the human tissue transglutaminase clone by in vitro translation. The clone encoded a
Mouse and Human Tissue Transglutaminases

Polypeptide that comigrated with authentic human tissue transglutaminase and reacted with a monoclonal antibody to the guinea pig liver transglutaminase. The apparent molecular weight of the translated product, M, 85,000 (based on its mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis), is higher than its deduced molecular weight of the translated product, transglutaminase and reacted with a monoclonal antibody to the guinea pig liver transglutaminase. The apparent molecular weight of the translated product also catalyzed the calcium-dependent covalent conjugation of putrescine to dimethylcasein indicating that the clone encoded a catalytically active enzyme. These studies indicate that unlike Factor XIII post-translational modification of tissue transglutaminase is not a prerequisite for enzymatic activity.

Comparison of Tissue Transglutaminase Sequences—The first tissue transglutaminase to be extensively characterized was from the guinea pig liver. This enzyme was first purified by Folk and Cole in 1966 (18) and was cloned and sequenced by Ikura et al. in 1988 (5). We have cloned tissue transglutaminases from human endothelial cells and mouse macrophages. Comparison of the deduced amino acid sequences of the three enzymes gives some indication of the degree of species and tissue homologies of this class of enzymes. It is clear from this comparison that the amino acid sequences of the tissue transglutaminases are highly conserved. The overall degree of the homology between the human and mouse enzymes is greater than 84%, between human and guinea pig the homology is 81%. Most of the differences in the nucleotide sequences are attributable to single nucleotide changes, the majority of which are silent in terms of the amino acid sequence. There are some substantial differences in regions of the human and guinea pig enzymes. Between nucleotides 1114-1203 and 1008-1097 of the human and mouse nucleotide sequences, respectively, the deduced amino acid sequences are identical, however, the insertion of a cysteine nucleotide at position 1077 in the guinea pig sequence introduces a shift in reading frame that results in 15 amino acid residues quite different from the human or mouse sequence (Fig. 8A). Deletion of a guanosine nucleotide in the guinea pig sequence terminates the reading frame shift and results in re-establishment of homology between the three species. A second major difference is found in the second putative calcium-binding domain of the guinea pig transglutaminase. There appears to have been the insertion of a 12-nucleotide segment in the guinea pig enzyme that was not found in either the human or mouse enzymes (Fig. 8B). Ikura has suggested that this region may contribute to the calcium-binding properties of the guinea pig enzyme. The human and mouse enzymes have comparable calcium activation kinetics but lack this segment making it unlikely that this second calcium-binding domain contributes to the kinetic properties of the enzyme.

The kinetic properties of the tissue transglutaminases have been extensively characterized. The cloned enzyme that was not found in either the human or mouse enzyme that was not found in either the human or mouse enzyme. The high degree of homology in this region of the human, mouse, and guinea pig enzymes (49 of 51 residues) is identical between the three enzymes in this region) suggests that the active site of these three enzymes has been stringently conserved. A second functional feature of these enzymes is their activation by Ca2+. Ikura et al. (5) suggested that the region between codons 446 and 453 showed properties associated with a Ca2+-binding pocket. This region is also highly conserved in the human and mouse enzymes increasing the likelihood that this region is in fact that functional calcium-binding domain. Recent studies have reported that tissue transglutaminases are GTP-binding proteins that show GTPase activity and whose cross-linking activity can be inhibited by GTP. However, sequence comparison between the three transglutaminases and GTP-binding proteins shows no elements of the consensus GTP-binding site (30). The molecular basis for this GTP-binding activity remains to be established.

Comparison with Other Transglutaminases—The preceding analysis indicates that there is high homology between the different tissue transglutaminases. We were interested in whether this homology extended to other transglutaminases. The cloning of the human endothelial tissue transglutaminase allows the direct comparison of two human transglutaminases, tissue transglutaminase and subunit a of Factor XIII (3, 4). The overall homology between the two enzymes is not very high, sequence comparison at the amino acid level yielded a homology value of 41%. However, there were regions of marked homology interspersed with regions where the two polypeptides were completely different. The cloning of the human endothelial tissue transglutaminase has suggested that these enzymes are likely to contain highly conserved functional domains. The cysteine residue at codon 277 of the human amino acid sequence is in the active site of the enzyme. The high degree of homology in this region of the human, mouse, and guinea pig enzymes (49 of 51 residues) are identical between the three enzymes in this region) suggests that the active site of these three enzymes has been stringently conserved. A second functional feature of these enzymes is their activation by Ca2+. Ikura et al. (5) suggested that the region between codons 446 and 453 showed properties associated with a Ca2+-binding pocket. This region is also highly conserved in the human and mouse enzymes increasing the likelihood that this region is in fact that functional calcium-binding domain. Recent studies have reported that tissue transglutaminases are GTP-binding proteins that show GTPase activity and whose cross-linking activity can be inhibited by GTP. However, sequence comparison between the three transglutaminases and GTP-binding proteins shows no elements of the consensus GTP-binding site (30). The molecular basis for this GTP-binding activity remains to be established.
homology. It is interesting that the degree of homology between the regions of the rabbit epithelial transglutaminase that have been cloned and the corresponding region of human tissue transglutaminase (32%) is comparable to the homology between the corresponding regions of human tissue transglutaminase and Factor XIII (36%). The similarities between epithelial and tissue transglutaminase and the subunit of Factor XIII suggest that these enzymes were derived from a common ancestral gene.

**Endothelial Cell Transglutaminase**—Greenberg et al. (27) reported that cultured human and bovine endothelial cells contain high levels of transglutaminase activity and immunochemical studies, suggested that the endothelial cell enzyme cross-reacted with antibodies to tissue transglutaminase. We found that the human endothelial cell cDNA libraries contained tissue transglutaminase clones at high frequency (0.25 clones/10,000 plaques screened) and Northern blot analysis indicated that the enzyme was abundant in these cells. The striking similarity in the sequence of the endothelial cell enzyme and the enzyme from macrophages and liver confirms that this is an authentic tissue transglutaminase.

**Transglutaminase Expression in Mouse Tissue**—There has been considerable speculation on multiple isoforms of the various transglutaminases (1). This speculation has been based on the fact that Factor XIII has been detected as both an intracellular and an extracellular enzyme, tissue transglutaminase has been detected intracellularly, associated with membranes and possible on the outside of cells (32). In addition, multiple different molecular weights and evidence for the electrophoretic heterogeneity of the enzyme have been reported (27). These observations have suggested that alternative forms of the enzyme might be present in cells and tissues. Our studies do not support this possibility. Analysis of genomic DNA indicates that tissue transglutaminase is present as a single copy gene. Northern gel analysis of multiple tissues have identified a single transcript of 3.6 kilobases and sequence analysis of overlapping cDNA clones has given no evidence of sequence heterogeneity in transglutaminase transcripts. Furthermore, in vitro translation of the transglutaminase enzyme yields an active enzyme with electrophoretic mobility identical to that of the purified enzyme (17). Thus, all our results are compatible with the idea that tissue transglutaminase is the product of a single gene, encoded into a single transcript, and expressed in cells without significant post-translational modification.

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2. J. P. Stein, unpublished observations.
Mouse and Human Tissue Transglutaminases

Supplemental Material to Isolation and Characterization of DNA Clones for Mouse Macrophage and Human Endothelial Cell Tissue Transglutaminases**

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Experimental Procedures

DNA Cloning and Sequencing of Mouse Macrophage and Human Tissue Transglutaminase

Cloning of transglutaminase cDNAs from various sources was accomplished by subtractive hybridization and lambda ZAP expression cloning. The subtractive hybridization approach was used to identify differences in expression patterns between various cell types. The cDNA library was constructed from poly(A)+ RNA isolated from mouse macrophages and human endothelial cells. The cDNA was ligated into lambda ZAP vector and transfected into bacteria. The resulting plaques were screened for the presence of transglutaminase cDNAs by hybridization to a transglutaminase-specific probe. The positive plaques were isolated and purified, and the cDNA inserts were sequenced by the dideoxy sequencing method.

Cloning and Sequencing of Human Tissue Transglutaminase

A human tissue cDNA library was constructed from various sources, including normal tissue and tissue from disease states. The library was screened for the presence of transglutaminase cDNAs by hybridization to a transglutaminase-specific probe. The positive clones were isolated and purified, and the cDNA inserts were sequenced by the dideoxy sequencing method.

DNA Sequencing and Analysis

DNA sequencing was performed using the dideoxy sequencing method. The sequencing reactions were performed using an automated DNA sequencer, and the sequences were analyzed using sequence analysis software.

Fig. 1. Sequence analysis of the mouse transglutaminase cDNA (top) and human transglutaminase cDNA (bottom). The sequences were aligned using the ClustalW program, and the alignment was used to generate a multiple sequence alignment.

To further characterize the transglutaminase activity of the cDNA clones, the cDNAs were ligated into yeast expression vectors and transfected into yeast cells. The resulting yeast cells were screened for the presence of transglutaminase activity. The positive clones were isolated and purified, and the cDNA inserts were sequenced by the dideoxy sequencing method.

Fig. 2. Sequence analysis of the mouse transglutaminase cDNA (top) and human transglutaminase cDNA (bottom). The sequences were aligned using the ClustalW program, and the alignment was used to generate a multiple sequence alignment.

Fig. 3. Sequence analysis of the mouse transglutaminase cDNA (top) and human transglutaminase cDNA (bottom). The sequences were aligned using the ClustalW program, and the alignment was used to generate a multiple sequence alignment.

Protein expression analysis of RNA derived from control and stimulated macrophages was performed to assess the transglutaminase activity. The RNA was extracted from control and stimulated macrophages, and the transglutaminase activity was measured using a radiolabeled kinase assay. The activity was found to be increased in stimulated macrophages, indicating that the transglutaminase activity was induced by stimulation.

Results

The results of the experiments are summarized in Table 1. The transglutaminase activity was found to be increased in stimulated macrophages compared to control macrophages. The results suggest that the transglutaminase activity is induced by stimulation.

Table 1: Summary of Results

| Condition         | Transglutaminase Activity (units/mg protein) |
|-------------------|---------------------------------------------|
| Control            | 0.5                                         |
| Stimulated         | 1.0                                         |

Discussion

The results of this study indicate that transglutaminase activity is induced by stimulation. This finding is consistent with previous reports that transglutaminase activity is induced by stimulation in other cell types. The increased transglutaminase activity in stimulated macrophages suggests that this enzyme plays a role in the immune response to infection.

Conclusions

The results of this study indicate that transglutaminase activity is induced by stimulation. This finding is consistent with previous reports that transglutaminase activity is induced by stimulation in other cell types. The increased transglutaminase activity in stimulated macrophages suggests that this enzyme plays a role in the immune response to infection.
Mouse and Human Tissue Transglutaminases

To isolate the active site of human tissue transglutaminase we purified the enzyme from pooled red blood cells (17). The purified enzyme was incubated with radiolabeled glycine and the resulting transglutamated product was analyzed by reverse-phase HPLC (Fig. 4). The peak corresponding to the active-site region was eluted at the position 343-347. The radioactive peptide derived from mouse brain (14304-14310) has been identified.

Fig. 4. Electron micrograph of a mouse brain transglutaminase. The active-site region is eluted at the position 343-347. The radioactive peptide derived from mouse brain (14304-14310) has been identified.

Fig. 5. Activity of human tissue transglutaminase (HCTM). To determine whether the human tissue transglutaminase (HCTM) was active, the enzyme was incubated with radiolabeled glycine and the resulting transglutamated product was analyzed by reverse-phase HPLC (Fig. 6). The peak corresponding to the active-site region was eluted at the position 343-347. The radioactive peptide derived from mouse brain (14304-14310) has been identified.

Fig. 6. Activity of human tissue transglutaminase (HCTM). To determine whether the human tissue transglutaminase (HCTM) was active, the enzyme was incubated with radiolabeled glycine and the resulting transglutamated product was analyzed by reverse-phase HPLC (Fig. 6). The peak corresponding to the active-site region was eluted at the position 343-347. The radioactive peptide derived from mouse brain (14304-14310) has been identified.
Mouse and Human Tissue Transglutaminases

Northern Blot Analysis of Human and Mouse Transglutaminases mRNA

Our original purpose in cloning human and mouse tissue TGase was to prepare hybridization probes suitable for detecting tissue transglutaminase mRNA in tissue. Figure 7A shows the Northern blot analysis of RNA from human and mouse testicular cells probed with radiolabeled mRNA prepared from the insert of clone STG-1. A single band at approximately 1.8 kilobases was detected. Figure 7B shows the results of Northern analysis of RNA prepared from several mouse tissues (thymus, spleen, kidney, lung, heart, and liver). All tissues contained varying levels of the same single 1.6 kb transcript.

Fig. 6. Western blot analysis of the 5 g TGase translation product of clone STG-1. Lane 1, immunoreactive purified human erythrocyte transglutaminase; Lane 2, 5 g TGase translation product without mRNA; Lane 3, 5 g TGase translation product with purified mouse testis mRNA; Lane 4, 5 g TGase translation product using mRNA from clone STG-1; Lane 5, W138 cell extract.

Fig. 7A. Identification of human tissue transglutaminase mRNA by Northern blot. 10 μg of mRNA from HELVEC were analyzed. Hybridization was done as described in the Experimental Procedures. The 1.6 kb fragmen of transglutaminase mRNA was detected in each tissue.

Fig. 7B. Identification of human tissue transglutaminase mRNA by Northern blot. 10 μg of mRNA from each tissue were analyzed. Hybridization was done as described in the Experimental Procedures. The 1.6 kb fragment of transglutaminase mRNA was detected in each tissue.