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GTP Hydrolysis by Human Tissue Transglutaminase Homologue

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Human tissue transglutaminase homologue cDNA was expressed in *E. coli* to analyze the catalytic characteristics. The transglutaminase homologue was purified by immunoaffinity chromatography. Specificity of GTP binding by the homologue was demonstrated by photoaffinity labeling in the absence or presence of GTP-γ-S. The homologue had GTPase activity with an apparent K_m value of 1.8 μM, several-fold lower than the reported K_m values for the native tissue transglutaminase. GTPase activity was inhibited by guanine nucleotides in order GTP-γ-S>GDP>GMP. The higher GTPase activity of the homologue may be related to the signaling events function.

It has been suggested that α-1-adrenergic transmembrane-signaling in rat liver involves the receptor, G-protein, and phospholipase C (PLC)[1] (1). A novel M_r = 74,000 GTP-binding protein, termed Gh, from rat liver which coupled to the α-1-adrenoceptor and stimulated a membrane-bound PLC was identified (2,3). This G-protein is not a substrate for bacterial toxins such as cholera and pertussis toxins. Another GTP-binding protein termed Gh7 (M_r = 78,000) was isolated from bovine heart (4). Using the α-agonist-receptor-Gh ternary complex preparation and Gh antibody, a Gh family coupling to the α-adrenoceptor and having different molecular masses in different species was characterized (5). The stimulation of PLC activity by Gh occurred effectively at a low calcium concentration (2 μM), but the PLC itself required 100 times more calcium to become fully activated (6). Recently (7), the Gh protein was found to be tissue transglutaminase (R-glutaminylpeptide: amine γ-glutamyltransferase, EC 2.3.2.13) (TGase) as determined by cDNA sequence analysis and immuno-detection studies. Co-transfection experiments utilizing TGase (Gh) cDNA showed that the expressed protein mediates α-adrenergic-receptor stimulation of PLC (7).

TGases catalyse an acyl-transfer reaction between peptide-bound glutamine residues and primary amines, including the ε-amino group of lysine residues in protein. Purified human erythrocyte TGase was found to have GTPase activity (8). The human tissue TGase native isoform (Mr = 80,000) has been mutated by site-directed mutagenesis (8). The active-site cysteine 277 was converted to serine. This residue is essential for TGase activity (cross-linking) but not for GTPase activity in the TGase isoform (Mr = 80,000) as recently reported (8).

A novel tissue transglutaminase homologue (TGH) was first described by Fraij et al., (9) from human erythroleukemia cells (HEL). A second human tissue transglutaminase homologue (TGH2) from HEL cells was later found by Fraij et al., (10). The fact that mRNAs for these homologues are induced by retinoic acid with similar kinetics during HEL cell differentiation suggests that a common regulatory mechanism and/or a functional association might exist between these proteins. Normal human fibroblast cells (WI-38) express homologues at much lower levels (9,10). The deduced amino acid sequences of TGase, TGH and TGH2 (10) showed identity for the amino acids shared among all three species. The TGH and TGH2 isoforms differ by terminational insertion in

[1] Abbreviations: PLC, phospholipase C; Gh, GTP-binding protein; TGase, tissue transglutaminase; TGH, tissue transglutaminase homologue; HEL, human erythroleukemia cells; LB, Luria broth; TB, terrific broth; IPTG, isopropyl β-D-thiogalactoside; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride.

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the carboxyl-terminal domain of two exons coding for 8 and 63 amino acids residues, respectively (9,10). The additional amino acids generate glycine-rich areas homologous to the consensus GTP-binding sequences (11). In humans regulation of TGase function may occur through these isoforms.

Analysis of the kinetic properties of the TGH isoform requires enzyme purification. Purification of TGH from tissues is not possible, because of the TGH isoform's low expression in tissues. In this report mass-production of TGH in *E. coli* and the purification of the enzyme are described. TGH was found to display a higher GTPase activity than the native isoform (TGase).

MATERIALS AND METHODS

Materials. Bacterial expression vector pET-14b and *E. coli* BL21(DE3) were from Novagen. Guanosine 5′-0-(3-thio)triphospate tetralithium salt (GTP-γ-S), GTP, GDP, and GMP (sodium salt) and activated charcoal (HCl-washed) were from Sigma. [γ-^{32}P]GTP (10 Ci/mmol) and [α-^{32}P]GTP (10 Ci/mmol) were from Amersham. Luria Broth and Terrific Broth media were from Gibco-BRL. Centricon-30 was from Amicon and Immobilon-P transfer membrane was from Millipore.

Construction of human tissue transglutaminase homologue (TGH). To express human TGH in *E. coli*, cDNA encoding TGH2 was inserted downstream from a T7 promoter between *NcoI* and *XhoI* restriction sites of pET-14b. The pET-TGH construction was introduced into *E. coli* BL21(DE3). Cells containing the TGH-expression vector were grown in LB or TB with ampicillin (50 μg/ml) at 37°C. At 0.6 A_{595} nm, induction of expression was initiated by adding isopropyl β-D-thiogalactoside (IPTG), to a final concentration of 0.2 mM. Cells were harvested 90 min later and resuspended in 0.02 M Tris-HCl, pH 7.5, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluride (PMSF), 1 mM EDTA, and 0.5% Triton X-100.

Purification of recombinant human TGH. Bacterial cells were lysed by sonication at 4°C and centrifuged for 10 min at 15,000 × g. Supernatants were adjusted to 0.01 M NH_4HCO_3, pH 8 and loaded onto a QA-cellulose column (15 × 2.5 cm), equilibrated with 0.01 M NH_4HCO_3, pH 8, 0.2 mM DTT, 1 mM EDTA and 1 mM PMSF. The column was washed with 3 bed volumes of the same buffer, TGH isoform was eluted with a linear gradient from 0–0.5 M NaCl in the same buffer. A major peak of TGH protein was eluted at about 2.7 M NaCl as detected by Western blot analysis. The corresponding fractions were applied to an immunoaffinity column (12), equilibrated with 0.02 M Tris-HCl, 0.2 mM DTT, 1 mM EDTA, and 0.15 M KCl. The column was washed with 20 bed volumes of the same buffer containing 0.5 M NaCl. The TGH isoform was eluted with 0.02 M Na_2CO_3, pH 10.5, 1 mM EDTA, 0.4 mM DTT, and 2 M KCl. Pooled fractions (10 ml) were concentrated to 0.5 ml by ultrafiltration on Centricon-30 with a buffer exchange into 0.05 M Tris-HCl, pH 7.5, 0.2 mM DTT, and 0.15 M NaCl. Protein concentrations were determined by the method of Bradford (dye reagent, BIORAD).

GTPase enzymatic assay. The TGH activity was assayed by measuring the amount of ^{32}Pi released from [γ-^{32}P]GTP. The enzymatic assay was performed as described (13) with some modification. Enzyme (1 μg) was incubated at 37°C in 60 μl of the reaction mixture containing 0.02 M Tris-HCl, pH 7.5, 0.5 mM DTT, 5 mM $MgCl_2$, and 2 μM [γ-^{32}P]GTP. After the incubation for each time point, 0.7 ml of ice-cold 5% (w/v) charcoal in 50 mM NaH_2PO_4 was added. The amount of ^{32}Pi released was then estimated by counting the radioactivity of 0.5 ml of the clear supernatants. Blank values were determined in the absence of enzyme. Data points are the mean of two independent experiments.

Photoaffinity labeling of TGH isoform. Purified enzyme (1 μg/30 μl) was incubated at 37°C for 10 min together with 10 μCi of [γ-^{32}P]GTP in 5 mM $MgCl_2$, 0.5 mM DTT, 0.02 M Tris-HCl, pH 7.5. Each reaction mixture was irradiated with uv light (Stratalinker, Stratagen, Inc.) on ice for 30 min and then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 9% acrylamide minislab system (Idea Scientific). Proteins were transfered by electrophoresis to Immobilon-P membrane by a minigene blotter (Idea Scientific). Immunostaining was performed with rabbit anti-TGH and a peroxidase-conjugated anti-rabbit IgG detection kit (Bio-RAD). The dried membranes were then exposed to RX-Fuji X-ray film.

Amino acid sequence analysis. Inclusion bodies produced from cells containing pET-TGH were separated by SDS-PAGE and transferred to Immobilon-P membranes. One lane was cut and used for immunostaining and the remaining membrane was stained with 0.1% Coomassie Blue R-250 in methanol, water, acetic acid (5:5:1) for 1 min. Membrane was destained in 50% methanol and rinsed extensively with distilled water. The TGH band was excised and subjected to amino acid sequence analysis on an Applied Biosystem Model 477A pulsed-liquid sequencer equipped with an in-line Model 120A PTH Analyzer.

RESULTS AND DISCUSSION

Expression, purification and GTP binding of TGH. A bacterial expression system was used to produce TGH enzyme. The IPTG-induction was for only 90 min to reduce the production of insoluble inclusion bodies. Total bacterial protein electrophoretic patterns revealed the presence of a major TGH band of M_r = 63,000 as determined by its reaction with a polyclonal antiserum against human erythrocyte TGase, as shown by Western analysis (Fig 1, Lanes 1 and 3). Amino

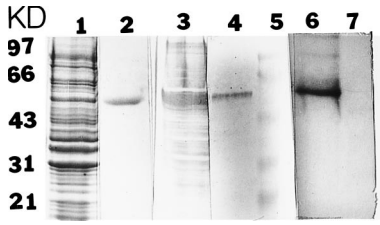

FIG. 1. Specificity of GTP binding by TGH: Sodium dodecylsulfate polyacrylamide gel electrophoresis, immunoblotting, and autoradiography. Coomassie Brilliant Blue (lanes 1, 2) and immunostaining (lanes 3, 4) of total bacterial proteins (lanes 1, 3) purified TGH isoform (lanes 2, 4). Autoradiography of purified TGH photolabeled with [α-^{32}P]GTP in the absence (lane 6) and presence (lane 7) of 0.1 mM GTP-γ-S. Lane 5 contains pre-stained molecular weight markers.

acid sequencing of recombinant TGH showed that the N-terminus had the same sequence present in the open reading frame as the reported cDNA (9). For fifteen cycles the amino acid sequence was AEELVLERCDLELET. The TGH isoform was purified as described under "Materials and Methods". The affinity-purified TGH isoform appeared as a single band with a M_r = 63,000 (Fig 1, Lanes 2 and 4). The purification of TGH was followed by measuring its enzymatic activity at 2 μM GTP. To determine the specificity of GTP binding to the TGH isoform, the [α-^{32}P]GTP photoaffinity labeling was evaluated. The binding ability of the TGH isoform was demonstrated to be specific as shown by the effective inhibition of labeling by the non-hydrolyzable analog of GTP, GTP-γ-S (Fig 1, Lanes 6 and 7). To further verify the GTP binding to TGH the purified enzyme was found to bind effectively to GTP-agarose column (data not shown) as described for the guinea pig liver transglutaminase purifications (14).

GTPase activity and kinetic parameters of TGH isoform. The intrinsic GTPase activity of TGH and its ability to hydrolyze GTP to GDP and phosphate was further characterized. As shown in Fig 2, incubation of purified TGH with [γ-^{32}P]GTP resulted in release of ^{32}Pi. This release was linear for up to 45 min with time and amount of the TGH enzyme. Metal ions were tested for their effects on the GTPase activity of TGH. The rate of the enzyme reaction increased in the presence of 5 mM $MgCl_2$ which was an optimal concentration for GTPase assays (13,15). When 5 mM $CaCl_2$ was used, there was no activation, but 5 mM $CaCl_2$ inhibited the Mg^{++} ion activation of GTPase activity by 50%. This suggests that Ca^{++} ion competes against Mg^{++} ion as an activator of the GTPase activity. Similar observations for Ca^{++} inhibition of GTPase activity were reported (14). The effect of increasing GTP concentrations on rates of GTP hydrolysis (Fig 3) revealed an apparent K_m of 1.8 μM which was in the range of K_m (0.3 μM) for typical GTPases (16), and was about 8-fold lower than K_m (14 μM) of human erythrocyte TGase, M_r = 80,000 isoform using the same assay

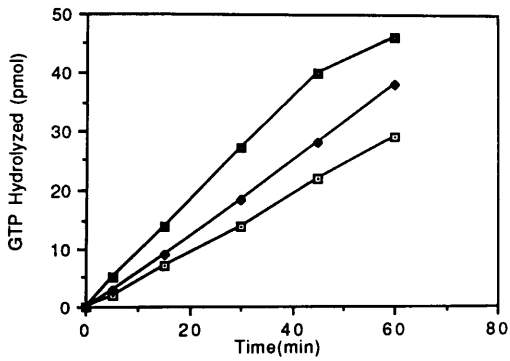

FIG. 2. Time course of [γ-^{32}P]GTP hydrolysis by purified TGH isoform. Assays were performed for each time point as described under "Materials and Methods." (□) 0.25 μg, (◆) 0.5 μg, (■) 0.75μg of pure TGH isoform.

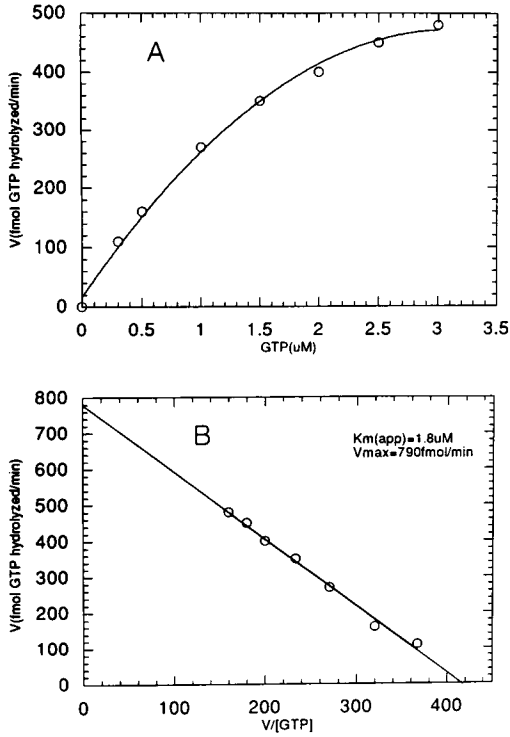

FIG. 3. GTP dependence of TGH isoform activity. GTPase was measured at varying GTP concentrations, as described under "Materials and Methods" using 0.25 μg of pure TGH enzyme. (A) Velocity as a function of GTP concentration. (B) Eadie-Hofstee plots of data presented in A. The K_m and the V_{max} were derived from the Eadie-Hofstee.

conditions reported earlier (8). The GTPase property of the TGH isoform was demonstrated to be specific as shown by the strong inhibition by various nucleotides (Fig 4). The order of inhibition of nucleotides for GTPase activity by TGH was GTP-γ-S>GDP>GMP.

These results indicate that TGH isoform is a specific GTP-binding protein. TGH was found as a product of alternative splicing events and expressed at much higher levels in tumor cells than normal cells. Investigation of TGase putative binding sites for GTP (11) showed that amino acid residues 520–540 contain the highest nucleotide-binding ability. This sequence is found in the main

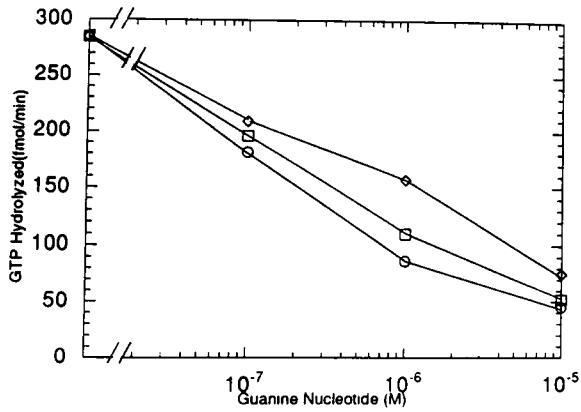

FIG. 4. Effect of guanine nucleotides on TGH isoform activity. Increasing concentrations of GTP-γ-S (○), GDP (□) and GMP (◇) were incubated with pure TGH enzyme (0.25 μg) for 60 min as described under "Materials and Methods."

TGase and in the TGH isoforms. However, TGH isoform contains the high affinity GTP binding site (520–540), and the alternative splicing events introduced one glycine at position 539 (9). It is interesting to note the K_m value of the purified human TGH isoform produced in *E. coli* is significantly lower than the K_m of purified human erythrocyte TGase isoform (8), and lower than the K_m of purified guinea pig liver transglutaminase (14). Whether these characteristics reflect a role in the signaling function of the TGH isoform as a GTP-protein remains to be shown.

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