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Putative nucleotide binding sites of guinea pig liver transglutaminase

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Three peptides corresponding to glycine-rich internal sequences of the guinea pig liver transglutaminase molecule were synthesized. These were peptide 1 (amino acid residues 520–544), peptide 2 (amino acid residues 345–367) and peptide 3 (amino acid residues 45–69). All of the synthetic peptides demonstrated significant binding ability for both ATP and GTP. Peptide 1 was the best protector of transglutaminase activity from both ATP and GTP inhibition, while peptides 2 and 3 protected the activity only from GTP inhibition. The data shown here lead us to propose putative binding site(s) for ATP and GTP guinea pig liver transglutaminase.

Transglutaminase; Nucleotide binding site

1. INTRODUCTION

Transglutaminase (*R*-glutaminylpeptide:amine γ-glutamyltransferase; EC 2.3.2.13) catalyzes an acyl-transfer reaction between peptidyl glutamine residues and primary amines including the ε-amino group of lysine residues in protein [1,2]. The enzyme distributes widely in cells, tissues, body fluids and even in some bacteria [1,3,4]. Plasma transglutaminase (Factor XIII) and epidermal transglutaminase are known to be involved in blood coagulation [5–7] and formation of the cornified envelope in epidermal keratinocytes [8], respectively. The physiological function of the tissue or cellular type enzyme still remains unresolved. It has, however, been postulated to participate in regulation of growth, differentiation and apoptosis of various cell types [9–14].

Recently two groups reported guanine nucleotides bind and inhibit tissue type transglutaminase from guinea pig liver and human erythrocyte [15,16]. We reported that the same enzymes demonstrated GTP hydrolysis activity [17]. We found that guinea pig liver enzyme hydrolyzed ATP as well (manuscript in preparation). Thus, it is significant to determine the site(s) for nucleotide binding to better understand the structure-function relationship of tissue transglutaminase.

We hypothesize that internal sequences in guinea pig liver transglutaminase molecule (amino acid residues 520–544, 345–367, and 45–69; [18]) are possible nucleotide binding sites because of their high glycine contents (see Table I and [19–23]). To test this hypothesis, we synthesized peptides corresponding to these amino acid residues. The synthetic peptides blotted to nitrocellulose membrane-bound ^{32}P-labeled ATP and GTP. Furthermore, the first peptide with amino acid residues 520–544 protected the transglutaminase activity from both ATP and GTP inhibition, while the second and third peptides showed their protective effect only on GTP inhibition. These observations suggest that the sequences are related to nucleotide binding. The results described here, in conjunction with previous reports [15–17], suggest an important physiological role of transglutaminase whose function is directly regulated by nucleotides.

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2. MATERIALS AND METHODS

ATP and GTP were purchased from Sigma. [α-^{32}P]ATP and [α-^{32}P]GTP were purchased from New England Nuclear. Guinea pig liver transglutaminase was purified to homogeneity from a commercial source (Sigma) by GTP-agarose affinity chromatography, as described [17]. The purified enzyme was stored in aliquots at −70°C. Protein concentrations were determined by the Bradford method [24].

The transglutaminase activity was measured for 20 min at 37°C in 0.2 ml of 40 mM Tris-HCl (pH 7.5)/150 mM NaCl (TBS) in the presence of 200 μM $CaCl_2$ and 40 μM EDTA (thus ~160 μM free Ca^{2+}) and 10 mM dithiothreitol as reported [25] using 1 mM [1,4-^{14}C]putrescine (spec. act. 1 μCi/μmol; Amersham) and 0.5 mg *N,N*-dimethylated casein (Calbiochem). ATP and GTP were present in the assay mixture at the indicated concentrations. The time course of the reaction was linear up to 40 min.

Based on the cloned sequence reported [18], peptides with internal sequences of guinea pig liver transglutaminase were synthesized by Dr. K. Jackson (Saint Francis Hospital of Tulsa, Medical Research Institute, University of Oklahoma, Oklahoma City) by F-moc peptide synthesis strategy [26] utilizing a DuPont ramps system and were purified by reverse-phase HPLC on a column of Synchropak RP-P C-18 (22.5 × 250 mm, Synchrom Inc., Linden, IN) using a linear gradient of 0.1% trifluoroacetic acid in H_2O and 0.08% in acetonitrile [27]. Peptides synthesized and used in this study are listed in Table I. The aqueous solution of synthetic peptides was adjusted to pH ~7.5 with Trizma base and HCl.

To detect their nucleotide binding activity, 10 nmol of each peptide

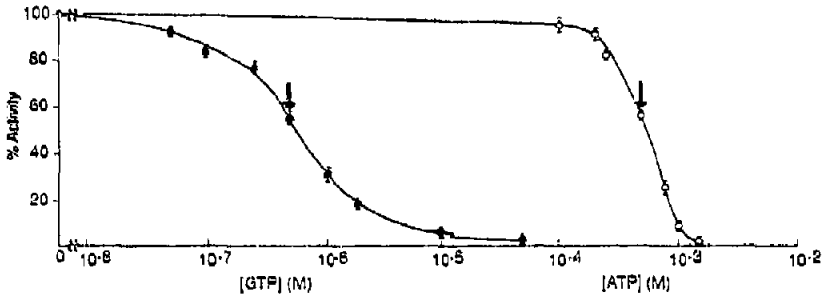

Fig. 1. Nucleotide inhibition of the transglutaminase activity. Transglutaminase activity was assayed with 0.28 μg enzyme in the presence of 200 μM $CaCl_2$ and 40 μM EDTA (thus ~160 μM free Ca^{2+}) plus various concentrations of GTP (●) or ATP (○). Values represent mean ± S.E.M. from 4–8 experiments. The abscissa of Figs. 1 and 2 are in logarithmic scale. Arrows indicate the concentrations of GTP (0.5 μM) and ATP (500 μM) that were used in the experiments for Table II and Fig. 2.

were spotted onto nitrocellulose membrane (Schleicher & Schuell) which was prewetted in H_2O. In order to minimize the non-specific binding of nucleotides to the membrane, the membrane was preincubated with 3% (w/v) BSA in TBS for 3 h at room temperature and was further incubated at 37°C for 30 min in 5 ml of TBS containing 500 μM [^{32}P]ATP (spec. act. 100 μCi/μmol) or 0.5 μM [^{32}P]GTP (spec. act. 2,000 μCi/μmol), and 200 μM $CaCl_2$/40 μM EDTA (+ Ca condition) or 40 μM EDTA (− Ca condition). The nitrocellulose membrane was then washed 3 times with 5 ml of ice-chilled, corresponding buffer which did not contain BSA or nucleotides. The membrane was dried in air and measured for radioactivity. Purified guinea pig liver transglutaminase (10 μg) was processed in a similar manner.

To test the ability of synthetic peptides to protect the transglutaminase activity from nucleotide inhibition, each peptide was preincubated with ATP or GTP for 10 min at 37°C in 90 μl of TBS (40 mM Tris-HCl/150 mM NaCl, pH 7.5) containing Ca^{2+}. Following the addition of enzyme (0.28 μg) and substrates ([^{14}C]putrescine plus dimethylcasein), the mixture was assayed for transglutaminase activity in 200 μl of TBS [25]. Final concentrations of free Ca^{2+}, ATP and GTP were 160 μM, 500 μM and 0.5 μM, respectively, and those of the peptides are indicated in Fig. 2. None of the peptides possessed any transglutaminase activity or were substrates for transglutaminase.

3. RESULTS

GTP was reported to be a potent inhibitor of tissue transglutaminase [15,16]. Fig. 1 illustrates that ATP is also inhibitory to guinea pig liver transglutaminase but at ~10^3-fold higher concentration compared to GTP (IC_{50}s of ATP and GTP were ~5.3×10^{-4} M and ~5.7×10^{-7} M, respectively). Guinea pig liver enzyme hydrolyzes GTP [17] as well as ATP (manuscript in preparation, see Introduction). Thus, the enzyme should contain nucleotide binding site(s). While the amino acid sequence deduced from cloned cDNA [18] did not represent any typical consensus motif for nucleotide binding, such as -G-X-G-X_2-G- [19–21] or G-X_4-G-K [22,23] (Xs are nonconservative residues, subscipts are the number of intervening residues, and the third Gly of -G-X-G-X_2-G- may be replaced by Ser or Ala in some protein kinases), we selected internal sequences of guinea pig liver enzyme for possible nucleotide binding sites based on glycine-rich regions. Peptides corresponding to these sequences were synthesized as listed in Table I.

Table II demonstrates that peptides 1, 2 and 3 which carried glycine-rich sequences bound both ATP and GTP. Peptide 4, a glycine-rich peptide based on the cDNA clone of human endothelial transglutaminase [28], also showed nucleotide binding. In contrast, peptide 5 which carried no such sequence showed no binding of the nucleotides. The ability of synthetic peptides to bind nucleotides was not related to their content of charged amino acids. However, Ca^{2+} decreased both ATP binding and GTP binding to peptide 2 ($P < 0.01$ in Student's t-test), perhaps suggesting another regulatory role of Ca^{2+} in the biochemical process which transglutaminase is involved in.

The nucleotide binding ability of the intact enzyme molecule was much greater than that of its partial pep-

Table I

Peptides synthesized and used in this study

Peptide*	Amino acid sequence**	Position in parental molecule
1	N-G-V-L-G-P-V-C-S-T-N-D-L-L-N-L-T-L-D-P-F-S-E-N-S	520–544
2	A-G-P-G-A-W-V-R-G-V-Q-A-L-D-P-T-P-Q-E-K-S-E-G	345–367
3	E-G-R-G-Y-E-A-G-V-D-T-L-T-F-N-A-V-T-G-P-D-P-S-E-E	45– 69
4	L-K-N-A-G-R-D-C-S-R-R-S-S-P-V-Y-V-G-R-V-G-S-G-M	204–227
5	K-I-S-T-K-S-V-G-R-D-E-R-E-D-I-T-H-T-Y-K	424–443

*Peptides 1–3 and 5 are deduced from cDNA sequence of guinea pig liver transglutaminase [18], while peptide 4 is deduced from cDNA sequence of human endothelial transglutaminase [28]. Peptide 5 was selected as a control.

**Single letter code was employed. The underlined amino acids are suggested to be key residues for possible nucleotide binding [19–23].

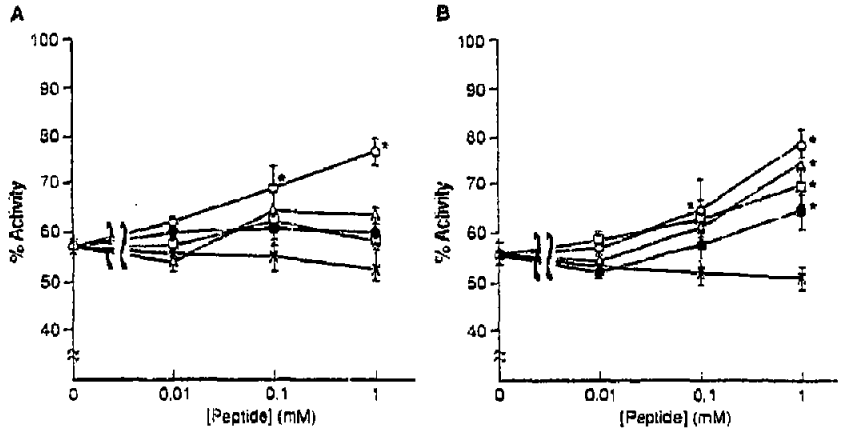

Fig. 2. Protection of the transglutaminase activity from nucleotide inhibition by synthetic peptides. Peptide 1 (○), 2 (●), 3 (□), 5 (×) and peptide 4 (△) were corresponding to amino acid residues 520–544, 345–367, 45–69 and 424–443 of guinea pig liver transglutaminase [18] and residues 204–227 of human endothelial transglutaminase [28], respectively. Activities obtained from 4–6 experiments were plotted as % of control assay which contained neither nucleotide nor synthetic peptide. The figure shows the recovery of transglutaminase activity by peptides from 500 μM ATP inhibition (A) and 0.5 μM GTP inhibition (B), respectively. *Significantly different ($P<0.02$, Student's t-test) from no peptide sample.

tides (Table II), probably because of the sustained higher structure of nucleotide binding sequence in the intact enzyme.

Since both GTP and ATP inhibited the transglutaminase activity of guinea pig liver enzyme (Fig. 1) and synthetic peptides which encompassed glycine-rich regions of the enzyme molecule were capable of binding nucleotides (Table II), we supposed that these peptides could release the transglutaminase activity from nucleotide inhibition. As shown in Fig. 2, peptide 1 significantly protected the transglutaminase activity from both ATP and GTP inhibition, while peptides 2 and 3 gave protection only against GTP inhibition. Peptide 5 which contained no glycine-rich sequence had no effect on GTP and ATP inhibition. The concentration of the synthetic peptide (1 mM, the molar ratio to the enzyme was 57,000:1) required for an efficient protection may indicate some additional sequence is also involved in nucleotide binding in the intact enzyme molecule (see Discussion). It is interesting to note that peptide 4 derived from endothelial transglutaminase [28] protected guinea pig liver enzyme activity from GTP inhibition (Fig. 2B). This suggests that the endothelial enzyme is also regulated by GTP.

Table II

Nucleotide binding to peptides

Nucleotide	Peptide	Nucleotide bound (fmol/nmol peptide)	
		+Ca	−Ca
ATP	1	452 ± 38	351 ± 17
	2	275 ± 4	156 ± 26*
	3	343 ± 9	335 ± 40
	4	228 ± 11	245 ± 6
	5	37 ± 9	10 ± 28
	Purified transglutaminase	64,260 ± 4,680	56,170 ± 1,280
GTP	1	25.3 ± 1.1	24.7 ± 1.6
	2	15.3 ± 0.5	6.4 ± 2.0**
	3	20.0 ± 1.4	21.4 ± 1.8
	4	13.5 ± 1.3	15.6 ± 1.5
	5	−2.5 ± 0.4	1.0 ± 1.2
	Purified transglutaminase	3,690 ± 251	4,081 ± 773

Values were expressed as mean ± S.E.M. from 4–6 experiments. Student's t-test between a value for peptide 5 and values for any other peptides demonstrated a statistical difference with $P<0.001$, with two exceptions under a $-Ca^{2+}$ condition as designated by * and ** (*$P<0.01$ between peptide 2 and 5; **no significant difference between peptide 2 and 5). * and ** also indicate that a $-Ca^{2+}$ condition decreased both ATP and GTP binding to peptide 2 ($P<0.01$, between +Ca and −Ca samples). Values cannot be compared between ATP and GTP, since different nucleotide concentrations (500 μM ATP and 0.5 μM GTP) were employed in this experiment according to their different inhibitory mode as shown in Fig. 1.

4. DISCUSSION

Peptides that contained glycine-rich sequences [19–23] were synthesized to study the nucleotide binding sites in guinea pig liver transglutaminase molecule (Table I). All such peptides demonstrated ATP and GTP binding, while peptide 5, which did not carry a glycine-rich sequence, showed no nucleotide binding (Table II). Among these peptides, peptide 1 was the best at protecting against both ATP and GTP inhibition (Fig. 2). This coincides with the highest nucleotide binding ability of peptide 1 in the presence of Ca^{2+}, compared to that of other peptides (Table II, and note that transglutaminase activity was assayed in the presence of Ca^{2+}). Peptides 2–4 protected the enzyme activity from GTP inhibition, but not from ATP inhibition, while

peptide 5 with no glycine-rich sequence did not show any protection against ATP or GTP inhibition (Fig. 2). Although none of the peptides match well with the canonical consensus motif for nucleotide binding [19–23], Table II and Fig. 2 suggest that peptides 1–4 which have glycine-rich sequences are related to the internal sequences for nucleotide binding. Thus, we propose that guinea pig liver transglutaminase contains at least three nucleotide binding sites, which correspond to peptides 1–3 listed in Table I.

When nitrocellulose membrane was not blocked with BSA prior to ATP binding assay, the experimental errors were large compared to those shown in Table II. However, ATP binding to peptides 2–4 was decreased to one-fifteenth, one-third and one-fifth, respectively, of that seen with peptide 1. This may explain the failure of peptides 2–4 to recover the ATP-inhibited transglutaminase activity (Fig. 2A, and note that BSA was not included in the enzyme assay). GTP binding to peptides was not significantly influenced by omitting the BSA blocking step (data not shown).

It is interesting to note that the amino acid sequence of guinea pig liver enzyme deduced from cDNA clone [18] shows DDQG (residues 231–234) and DQAG(residues 343–346) that match with the second consensus motif for nucleotide binding (element II, designated as DX_2G; [29]), while a sequence DAPIG (residues 108–112) is element II-like. One of these sequences may couple with residues 45–69 (peptide 3) in nucleotide binding.

We have proposed possible ATP and GTP binding sites based on the data shown in Table II and Fig. 2. However, these sites still remain to be established, since it is important to determine their affinities for nucleotides in the intact enzyme molecule. Ca^{2+} appears to be an additional factor in this regard, as the divalent cation modified the nucleotide binding ability of peptide 2 (Table II). It is also important to determine which nucleotide site is involved in the nucleotide hydrolysis activity of guinea pig liver transglutaminase [17]. Various approaches must be taken to clarify the structure–function relationship of this multifunctional enzyme and its regulation.

After this work was completed, purified rat liver transglutaminase was reported to be inhibited more effectively by ATP than by GTP [30], contrary to the previous reports [15,16] as well as this report (Fig. 1). Tissue type transglutaminase from different sources may have different sensitivity to nucleotides.

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