Two distinct classes of rat intestinal mucosal enzymes incorporating putrescine into protein

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Abstract Tissue-transglutaminase (t-TGase) is a family of calcium-dependent enzymes. A Ca^{2+}-independent soluble enzyme, in addition to t-TGase, capable of incorporating polyamines into proteins was demonstrated in rat intestinal mucosa. The Ca^{2+}-independent enzyme was stimulated 2- to 5-fold by Fe^{2+} and Co^{2+} ions but inhibited by Cu^{2+} and Zn^{2+} ions. The Ca^{2+}-stimulated t-TGase activity was inhibited by divalent ions in the following order: Zn^{2+}, $Fe^{2+} > Co^{2+} > Cu^{2+}$. The opposite effects of EGTA, Fe^{2+} and Co^{2+} on these two enzyme activities indicate that they are two distinct classes of enzymes. Competition studies demonstrated differential preferences of the two enzymes for substrates. The Ca^{2+}-dependent enzyme preferred putrescine, monodansylcadaverine > cadaverine, spermidine, spermine > 1,10-diaminodecane > triethylbutylamine. On the other hand, the Ca^{2+}-independent enzyme preferred putrescine > cadaverine > spermine, 1,10-diaminodecane > spermidine > monodansylcadaverine > triethylbutylamine. Further studies with divalent ions excluded the possible association of this novel Ca^{2+}-independent enzyme with diamine oxidase. Finally, the Ca^{2+}-independent enzyme had a higher affinity for putrescine (K_m = 0.02 mM) than did Ca^{2+}-dependent t-TGase (0.2 mM). As judged by gel filtration on HiPrep Sephacryl 200 column, the Ca^{2+}-independent enzyme had a molecular weight of ~48 kDa, the intestinal Ca^{2+}-dependent t-TGase was about 188 kDa while that of testicular t-TGase was about 96 kDa. In conclusion, the Ca^{2+}-independent enzyme is stimulated by cobalt or ferric ions, and selectively incorporates aliphatic diamines or polyamines with symmetric amino groups. The observed Ca^{2+}-independent enzyme activity is not related to diamine oxidase or its products. With a 10 times greater affinity for putrescine, the calcium-independent, 48-kDa intestinal enzyme may mediate polyamine function better than calcium dependent, 188-kDa intestinal tissue transglutaminase in the intestinal mucosa.

Key words: Intestine; Mucosa; Transglutaminase; Polyamine; Putrescine; Diamine oxidase

1. Introduction

Transglutaminase (TGase), a Ca^{2+}-dependent enzyme, catalyzes the formation of ε-(γ-glutamyl)lysine crosslinking of protein or peptide molecules [1,2]. Several species of TGases have been documented: blood coagulating factor XIII, epidermal insoluble TGase, soluble tissue transglutaminase (t-TGase), etc. The involvement of factor XIII in blood coagulation [1] and epidermal particulate-bound TGase in keratinocyte differentiation [3–5] are well established. However, t-TGases are reported to be associated with cell proliferation [6,7], cell differentiation [7–9], receptor-mediated endocytosis [1,7,10,11], cell senescence [12], programmed cell death [7,13], intracellular matrix [7,14], and aging [2,15]. Recently, the induced expression of t-TGase was reported to be one of the effector elements of apoptosis pathway [16–19]. The increased t-TGase may be required in the dying cells to ensure the appropriate completion of biochemical and morphological processes of apoptosis due to its function for covalent crosslinking of cellular proteins [17,19,20].

In addition to the role of t-TGase in the molecular pathway of apoptosis, data documented in the last few years also demonstrated that t-TGase is a new class of GTP-binding protein (Gh). The Gh participates in receptor signaling and may be a component of a complex regulatory network in which receptor-stimulated GTP binding switches the function of Gh from t-TGase to receptor signaling [21–24]. When the last 149 amino acid residues near the C-terminus of t-TGase were deleted, its crosslinking activity was reduced but magnesium-dependent GTP/ATPase activity was enhanced [24]. However, eight amino acid residues near the t-TGase C-terminus were shown to be critical for recognition and stimulation of phospholipase C [25]. Furthermore, G-protein activity of the t-TGase but not its crosslinking activity was reported to play a role in cellular progression from S-phase to G2/M phase during cell cycle [26].

Putrescine and polyamines (spermidine and spermine) have been shown to be natural substrates for TGase, to form γ-glutamylamine derivatives, and to be conjugated with cellular proteins. Both non-crosslinked and crosslinked products are formed. The diamine- and polyamine-modified proteins have been found intra- [14,27,28] and extra-cellularly [29]. These aliphatic diamines and polyamines are important factors or mediators in gastrointestinal adaptation [30–33] and healing [34,35]. The inhibition of t-TGase-induced protein crosslinking with dansylcadaverine prevented the effect of polyamines on the healing of gastrointestinal mucosa [36]. This suggests that t-TGase may play a role in mediating polyamine function during healing and in maintaining the normal physiological function of gastrointestinal mucosa. It is interesting to note that TGF-β was reported earlier to induce apoptosis [37] while polyamines regulate TGF-β expression during gastrointestinal wound healing [38]. Consequently, the essentiality of polyamines and t-TGase in early healing of gastrointestinal mucosal erosion may be related to apoptosis of injured cells.

Further studies on gastrointestinal mucosal t-TGase led to the discovery of a novel Ca^{2+}-independent, TGase-like enzyme in addition to the Ca^{2+}-dependent t-TGase in all regions of rat intestinal mucosa [39]. In this paper, the properties of these two distinct classes of enzymes were further characterized and compared.

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2. Materials and methods

2.1. Materials

Adult Sprague-Dawley rats were housed at 22°C with a 12-h light-dark cycle and allowed free access to tap water and standard laboratory rat food.

Putrescine dihydrochloride-[2,3-^{3}H(N)] (specific activity 40.3 Ci/mmol) was from New England Nuclear (Boston, MA, USA). Putrescine dihydrochloride, cadaverine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, and monodansylcadaverine hydrochloride were purchased from Sigma (St. Louis, MO, USA). Monodansylcadaverine hydrochloride (100 mM) was first dissolved in 10% acetic acid and then diluted to the desired concentrations.

2.2. Methods

2.2.1. Sample preparation and transglutaminase assay. Distal ileum mucosal tissue was collected and homogenized in 10 volumes (v/w) of 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA and 5 mM dithiothreitol (DTT). The 30 000×*g* (45 min) supernatant was transferred to a new tube for subsequent TGase assay and protein analysis. TGase activities were determined in a 50-μl reaction mixture. The procedure of Murtaugh et al. [40] was followed with modification [39]. Each reaction mixture containing 0.5–4 μCi [^{3}H]putrescine at 0.25–250-μM concentrations, 2 mg/ml dimethylcasein, 5 mM (or as stated) $CaCl_2$ or EDTA, 50 mM Tris-HCl, pH 9.0, and 30 μl of 30 000×*g* supernatant was incubated at 37°C for 15 min. The incubation was ended by adding an equal volume (50 μl) of ice-cold 0.25 M putrescine and mixed. An aliquot of 80 μl was spotted onto a strip of 3 MM Whatman filter paper and plunged immediately into ice-cold 10% trichloroacetic acid (TCA) for 30 min. After washing 3 more times in ice-cold 5% TCA followed by 3 rinses with ice-cold 95% ethanol, the air dried filter was counted in 5 ml of scintillation fluid (EcoLite(+), ICN Biomedical, Irvine, CA, USA) with a Beckman LS 5000TA (Fullerton, CA, USA). The blanks were performed by adding 50 μl ice-cold 250 mM putrescine immediately prior to the addition of enzyme to the reaction mixtures. Protein concentration of 30 000×*g* supernatant was determined by the Bradford method [41]. Enzymatic activities were expressed as cpm incorporated per mg protein per min, or ρmol incorporated per mg protein per h. Each datum represents mean ± S.E., $n = 3$ or 4 determinations.

2.2.2. The effect of divalent ions on transglutaminase activities. To study the effect of various divalent ions on the intestinal mucosal t-TGase activity, aliquots (30 μl) of 30 000×*g* supernatant were assayed in the presence or absence of 5 mM EGTA, of 5 mM divalent ion (Ca^{2+}, Co^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, or Mg^{2+}) or of 5 mM cystamine. Further experiments were performed by including 5 mM of each divalent ion in the presence of either 2 mM EGTA or 2 mM $CaCl_2$. The effect of divalent ions on thermal stability of the TGase was investigated by incubating aliquots of the 30 000×*g* supernatant with the indicated divalent ions at 55°C for 30 min prior to enzyme assays.

2.2.3. Diamine oxidase assay. Diamine oxidase (DAO) activity was determined by measuring the amount of Δ^1-[^{3}H]pyrroline formed [42]. The reaction mixtures contained identical components as those for TGase assays except dimethylcasein. At the end of the assay, an equal volume of 250 μM putrescine was added, mixed, and then subjected to Δ^1-[^{3}H]pyrroline extraction with 1 ml toluene. Aliquots of toluene extract were dried and counted for radioactivity.

2.2.4. K_m determinations. Aliquots of incubation mixtures containing various concentrations of [^{3}H]putrescine (0.1–4 mM) in separate assay tubes were prepared prior to the addition of 30 000×*g* supernatant. The reaction was performed at 37°C for 0, 0.5, 1, 2, 4, 6, and 8 min and terminated as described previously. The initial velocity (V_0) for each putrescine concentration ([S]) was determined first. The K_m value for each enzyme was subsequently determined by using Lineweaver-Burk double reciprocal plots, $1/V_0$ vs. 1/[S].

2.2.5. Gel filtration of ammonium sulfate precipitated enzymes. The 30 000×*g* supernatant of whole intestinal mucosa from 3 rats was fractionated with saturated ammonium sulfate at 45–55% saturation [39]. The precipitated material was collected at 10 000×*g* for 10 min. The pellet was redissolved in approximately 5 ml homogenizing buffer and dialyzed against 100× volumes of the same buffer overnight with two changes. The dialyzed sample was concentrated in a Millipore 10 KD concentrator (Ultrafree-15 Centrifugal Filtration Device) to less than 2 ml. The sample was clarified by filtration (with 0.45-μm filter) and then further purified on a Pharmacia HiPrep Sephacryl 200 column. Each 1-ml fraction was collected and assayed for transglutaminase activity either in the presence of 5 mM $CaCl_2$ or 5 mM EGTA. Molecular weight standards were used to determine V_0 and molecular weight standard curve for the determination of molecular weights of each enzyme peak.

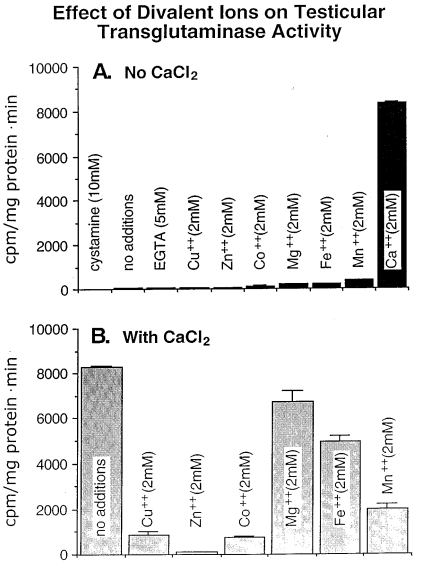

Fig. 1. Effect of divalent ions on testicular transglutaminase activity. Rat testicular cytosol was prepared and tissue transglutaminase activity determined as described in Section 2.2. A: Aliquots of cytosol were assayed in the presence of cystamine, EGTA, or divalent ions as indicated. 'No addition' means that enzyme activity was assayed in reaction buffer without additions as described above. B: Enzyme activity was measured in the presence of 2 mM $CaCl_2$ or 2 mM $CaCl_2$ plus divalent ions indicated.

3. Results and discussion

3.1. Effects of divalent ions on enzyme activities

Soluble rat testicular TGase was used as an example of a typical t-TGase whose activity was greatly stimulated by $CaCl_2$ (Fig. 1A). However, in the presence of 2 mM $CaCl_2$, the testicular t-TGase activity was markedly suppressed by the addition of other divalent ions in the following order: $Zn > Cu^{2+}$, $Co^{2+} > Mn^{2+} > Fe^{2+} > Mg^{2+}$ (Fig. 1B). On the

Table 1
Competitive inhibition of [^{3}H]putrescine incorporation by various polyamines

Agent included (1 mM)	Percent (%) of control	
	Assay in $CaCl_2$	Assay in EGTA
Control	100.0 ± 3.5	100.0 ± 1.4
Cystamine	20.4 ± 0.2	10.2 ± 0.4
Putrescine	25.2 ± 0.9	23.4 ± 2.0
Cadaverine	46.1 ± 1.4	33.0 ± 2.2
Monodansylcadaverine	29.4 ± 0.4	91.3 ± 5.0
Spermidine	43.4 ± 1.9	63.6 ± 4.2
Spermine	40.9 ± 1.5	43.1 ± 1.7
1,10-Diaminodecane	60.7 ± 1.4	44.9 ± 0.1
Triethylbutylamine	99.1 ± 4.7	95.6 ± 3.0
Acetic acid (0.1 mM)	103.0 ± 0.6	110.0 ± 6.6

The incorporation of [^{3}H]putrescine was performed in the presence of 1 mM of the indicated competitors. The reaction mixtures contained 250 μM putrescine, 5 mM $CaCl_2$ or EGTA as described in Section 2.2. The control values for 5 mM $CaCl_2$ and 5 mM EGTA are 1122 ± 39 and 695 ± 9.5 ρmol/h/mg protein, respectively. Data represent means ± S.E., $n = 3$.

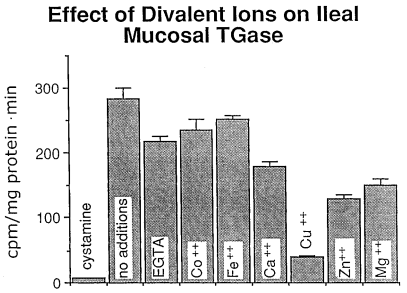

Fig. 2. Effect of divalent ions on transglutaminase activity in rat ileal mucosa. Ileal mucosa was homogenized in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 15 mM β-SHEtOH. Aliquots of sample were assayed for transglutaminase activity in the presence of either 10 mM cystamine, 5 mM EGTA or 2 mM divalent ions as indicated. Data represent mean ± S.E., $n = 3$.

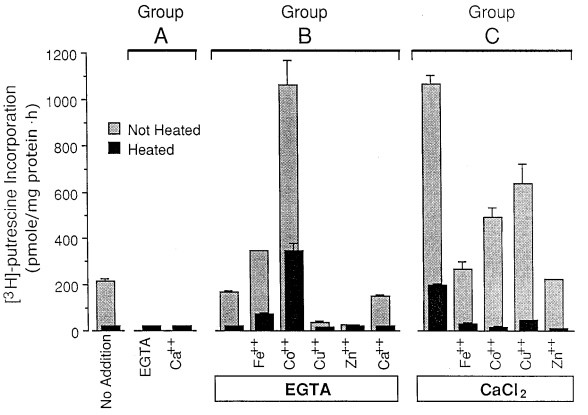

Fig. 3. Stability of ileal mucosal enzymes at 55°C. A: Enzyme was heated in homogenizing buffer first, then either 2.5 mM EGTA or divalent ion was included in enzyme assay. B: EGTA (2.5 mM) was first mixed with enzyme prior to the addition of other divalent ions (5 mM). The enzyme was then incubated at 55°C for 30 min in the presence of the indicated divalent ion followed by cooling at 0°C for 30 min prior to assay. C: $CaCl_2$ (2.5 mM) and the indicated divalent ion (5 mM) were mixed well before the addition of enzyme. The enzyme-divalent ion mixture was then treated as stated in B prior to enzyme assay. Data represent mean ± S.E., $n = 3$. Shaded and solid bars represent activities of non-heated and heated enzymes, respectively.

contrary, TGase activity of rat intestinal mucosa measured in the presence of 5 mM EGTA was not always less than that measured in 5 mM $CaCl_2$ (Fig. 2). As summarized in Fig. 3, the Ca^{2+}-independent t-TGase was stimulated by Fe^{2+} or Co^{2+} (2–5-fold). The magnitude of stimulatory effects of Fe^{2+} or Co^{2+} varied from rat to rat. Both Cu^{2+} and Zn^{2+} inhibited the Ca^{2+}-independent putrescine incorporation (Fig. 3). On the other hand, all divalent ions tested reduced the activity of Ca^{2+}-dependent t-TGase in the order as follows: Zn^{2+}, $Fe^{2+} > Co^{2+} > Cu^{2+}$ (Fig. 3). Both enzymes were thermally labile at 55°C (Fig. 3). However, Fe^{2+} and Co^{2+} partially stabilized the Ca^{2+}-independent TGase while Ca^{2+} partially stabilized the Ca^{2+}-dependent t-TGase. The addition of any one of the other divalent ions together with Ca^{2+} abolished the thermal stabilizing effect of Ca^{2+} on the Ca^{2+}-dependent t-TGase at 55°C.

3.2. *Competitive inhibition of [^{3}H]putrescine incorporation by polyamines*

The competitive inhibition of [^{3}H]putrescine incorporation was performed in the presence of 0.25 mM [^{3}H]putrescine. Four-fold excess of unlabeled (1 mM) putrescine reduced the incorporation of radioactivity into proteins catalyzed by both enzymes to approximately 20–25% of controls (Table 1). This is in accordance with the magnitude of isotope dilution. The relative effectiveness of various tested agents on the competitive inhibition of [^{3}H]putrescine incorporation by Ca^{2+}-dependent t-TGase was as follows: cystamine, putrescine, monodansylcadaverine > spermine, spermidine, cadaverine > 1,10-diaminodecane > triethylamine (Tables 1 and 2). Nevertheless, the Ca^{2+}-independent enzyme exhibited a different preference for substrates (Table 1). The inhibitory efficiency was as shown in the following sequence: cystamine > putrescine > cadaverine > spermine, 1,10-diaminodecane > spermidine > monodensylcadaverine > triethylamine. (Table 1).

The above data revealed several interesting properties of the two enzymes. Monodansylcadaverine (a non-crosslinker) was the best competitor, comparable to unlabeled putrescine (as predicted by isotope dilution), in the incorporation of [^{3}H]putrescine into proteins catalyzed by the Ca^{2+}-dependent t-TGase. However, in the case of the Ca^{2+}-independent enzyme, cadaverine, spermine and 1,10-diaminodecane were the best competitors. In fact, they competed better than spermidine did with [^{3}H]putrescine. Monodansylcadaverine did not compete with [^{3}H]putrescine in the case of Ca^{2+}-independent enzyme. The observations suggest that this novel enzyme interacts selectively with molecules containing two amino groups at symmetric positions even up to 10 carbon apart.

Some common characteristics were shared by these two enzymes. (i) The incorporation of [^{3}H]putrescine into proteins was suppressed by cystamine, $CuSO_4$, $ZnCl_2$, putrescine, cadaverine, spermine, 1,10-diaminodecane and so forth. (ii) Both exhibited an optimal pH at 9.0 [39], indicating the requirement that at least one of the amine functions be unprotonated. (iii) Incubation of the thermal labile enzyme preparations at 55°C abolished their activities under the present experimental conditions.

Intestinal mucosa is rich in diamine oxidase [43] which con-

Table 2
The effect of $CoCl_2$ on [^{3}H]putrescine incorporation and diamine oxidase activity

Assay conditions	[^{3}H]Putrescine incorporated (cpm/min/mg protein)	Δ^1-[^{3}H]Pyrroline produced (cpm/min/mg protein)
No addition (control)	173 ± 6.5	605 ± 62
+2.5 mM Co^{2+}	839 ± 21.9	510 ± 32.8
+5.0 mM Co^{2+}	948 ± 25.4	494 ± 8.3

Aliquots of 380 μg ileal mucosal cytosol were incubated in a 52.5-μl reaction mixture, pH 9.0, containing 250 μM [^{3}H]putrescine, with (for [^{3}H]putrescine incorporation) or without (for Δ^1-pyrroline formation, diamine oxidase activity) 105 μg dimethylcasein at 37°C for 15 min or 30 min, respectively. The Δ^1-[^{3}H]pyrroline was extracted with 1 ml toluene. The incorporation of [^{3}H]putrescine into protein was determined by 10% TCA-precipitable radioactivity bound to Whatman filter paper. Data represent means ± S.E.; $n = 3$.

verts [^{3}H]putrescine to [^{3}H]-γ-aminobutylaldehyde and subsequently to Δ^1-[^{3}H]pyrroline and/or [^{3}H]-γ-aminobutyric acid (GABA) or [^{3}H]-γ-succinate [44]. The possibilities that the Ca^{2+}-independent enzyme might incorporate catabolic metabolites of [^{3}H]putrescine produced by diamine oxidase or aldehyde dehydrogenase were investigated in a cell free system. The indifference of Ca^{2+}-independent enzyme with DAO was demonstrated by including various divalent ions separately in the assay. $CoCl_2$ stimulated the Ca^{2+}-independent incorporation of ^{3}H- substrate into proteins but failed to significantly stimulate the formation of Δ^1-[^{3}H]pyrroline catalyzed by DAO in the TGase assay buffer (Table 2). Furthermore, the production of Δ^1-[^{3}H]pyrroline by the copper containing DAO [45] was stimulated by Zn^{2+} and Cu^{2+}. On the contrary, both Zn^{2+} and Cu^{2+} markedly inhibited incorporation of radioactivity by Ca^{2+}-independent enzyme into TCA-precipitated molecules (Fig. 3, Table 3). It is apparent that the Ca^{2+}-independent enzyme showed markedly different characteristics from DAO. The data further demonstrated that the incorporated ^{3}H-material was not likely to be a catabolic product of [^{3}H]putrescine. However, the data does not exclude the possibility that the ^{3}H-higher polyamines or the metabolites of ^{3}H-higher polyamines were incorporated into casein.

3.3. The K_ms of Ca^{2+}-dependent and independent enzymes

The initial velocities of both TGases in various [^{3}H]putrescine concentrations were first assessed from enzyme activities obtained from the assays performed in each putrescine concentration for various times as described in Section 2.2. Fig. 4 shows the double reciprocal Lineweaver-Burk plots of Ca^{2+}-dependent and Ca^{2+}-independent enzymes using the calculated initial velocities and the respective substrate concentrations. The Ca^{2+}-dependent t-TGase exhibited an approximate K_m value of 0.20 mM while the Ca^{2+}-independent enzyme showed a K_m of 0.02 mM. The V_{max} values of both enzymes differed from rat to rat. The Ca^{2+}-independent enzyme exhibited about 10 times greater affinity for the [^{3}H]putrescine than did the Ca^{2+}-dependent t-TGase. The cellular levels of polyamines vary greatly from the crypt cells to the villus tip cells [30]. When polyamine concentrations are low (<250 μM), the incorporation of ^{3}H-polyamines into proteins by the Ca^{2+}-independent enzyme may become prominent [39]. Localization of both enzymes along the intestinal crypt to villus axis is crucial to understanding the function(s) of these two enzymes in intestinal physiology, development and healing.

Fig. 5 further demonstrated that the intestinal Ca^{2+}-independent enzyme showed a molecular weight of about 48 kDa while the Ca^{2+}-dependent t-TGase was about 188 kDa. On the other hand, the molecular weight of testicular t-TGase was estimated around 96 kDa (Fig. 5C), close to that reported by Seitz et al. [46]. Recent data have documented that t-TGase is also a GTP-binding protein [21]. The binding of GTP to

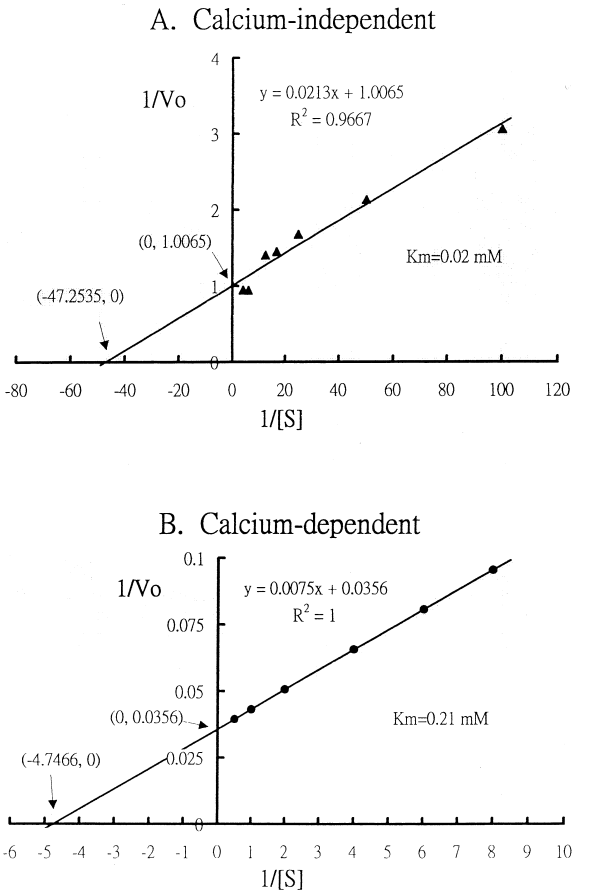

Fig. 4. Lineweaver-Burk plots of Ca^{2+}-dependent and Ca^{2+}-independent enzymes of rat ileal mucosa. The double reciprocal plots of the Ca^{2+}-independent (A) and the Ca^{2+}-dependent enzymes (B) are generated from the initial velocity of enzyme activity at various [^{3}H]putrescine concentrations as described in Section 2.2. The calculated K_m derived from A for the Ca^{2+}-independent enzyme is 0.021 mM and that from B for the Ca^{2+}-dependent enzyme is 0.21 mM. The V_{max}s are 1 pmol/mg protein/min and 28 pmol/mg protein/min for Ca^{2+}-independent and Ca^{2+}-dependent enzymes, respectively. The unit of [S] is mM and V_0 is pmol/mg/min.

t-TGase switches its function from transglutaminase to receptor signaling [21–24]. Whether or not this novel Ca^{2+}-independent enzyme is also a GTP-binding protein remains to be clarified.

In conclusion, a Ca^{2+}-independent enzyme, capable of incorporating putrescine or polyamines into proteins, is present in rat intestinal mucosa. This enzyme interacts preferentially with diamines or polyamines containing two discrete primary amino groups at symmetric positions. The non-crosslinker, monodansylcadaverine, does not significantly inhibit putrescine incorporation catalyzed by this enzyme as it did by the Ca^{2+}-dependent tissue transglutaminase. Exhibiting 10 times as much affinity for putrescine as Ca^{2+}-dependent tissue transglutaminase, this 48-kDa novel enzyme has the potential to

Table 3
The effect of $ZnCl_2$ and $CuSO_4$ on ileal mucosal transglutaminase and DAO activities

Assay condition	[^{3}H]Putrescine incorporated (ρmol/h/mg protein)	Δ^1-[^{3}H]Pyrroline produced (ρmol/h/mg protein)
EGTA (control)	1002 ± 65	1157 ± 75
+5 mM $ZnCl_2$	66 ± 3	1676 ± 228
+5 mM $CuSO_4$	88 ± 3	1536 ± 396

The transglutaminase and DAO activities were assayed as described in Table 2.

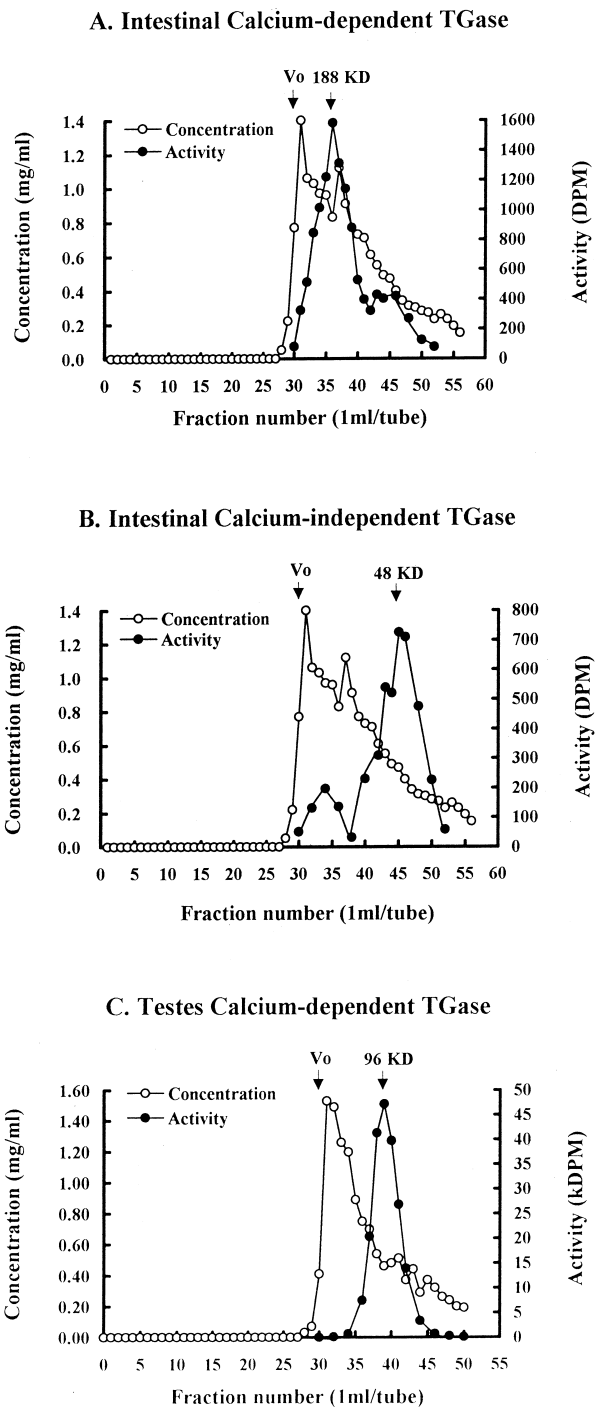

Fig. 5. Gel filtration of ammonium sulfate precipitated enzymes. All experimental procedures were described in Section 2.2. Solid circles represent enzyme activity while open circles show protein concentration in each fraction.

play a crucial role in mediating the physiological function of diamines and polyamines in rat intestinal mucosa, especially when substrates are present at low concentrations.

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