Calmodulin Regulates Nucleotide Hydrolysis Activity of Tissue Transglutaminase

Yutaka Takeuchi, Paul J. Birckbichler, Manford K. Patterson, Jr., Kyung, N. Lee and Henry A. Carter

The Samuel Roberts Noble Foundation, Inc., Biomedical Division, Ardmore, Oklahoma 73402, U.S.A.

Z. Naturforsch. **49 c,** 453–457 (1994); received March 17/May 16, 1994

Calmodulin, Transglutaminase, Nucleotide Hydrolysis

The interaction of calmodulin with purified guinea pig liver transglutaminase was studied. The nucleotide (ATP and GTP) hydrolysis activity of this tissue transglutaminase was transiently increased and then gradually decreased depending on calmodulin concentration. The peak activation was obtained in the presence of a stoichiometric amount of calmodulin. The effect of calmodulin on the classical transglutaminase activity was minimal. Fluorescence spectroscopy demonstrated that the enzyme produced a significant blue shift in the emission peak of dansylated calmodulin. Interestingly, Ca^{2+} was not required for the interaction between the two proteins. The results described here give an additional regulatory role to calmodulin.

Introduction

Calmodulin (CaM) is a ubiquitous Ca^{2+}-binding protein, which regulates various Ca^{2+}-related cellular processes (Klee and Cohen, 1988). Ca^{2+}-CaM complex is involved in such diverse reactions as the activation of phosphodiesterases, adenylate cyclases, protein kinases, phosphatases, Ca^{2+}-ATPases, and so forth. 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 proteins (Folk and Finlayson, 1977; Lorand and Conrad, 1984). The enzyme is distributed widely in cells, tissues, body fluids and even in plants and bacteria (Folk and Finlayson, 1977; Lorand and Conrad, 1984; Chung, 1975; Ando *et al.*, 1989; Margosiak *et al.*, 1990). The enzyme, with the exception of plants and bacteria, is Ca^{2+}-dependent (Lorand and Conrad, 1984; Chung, 1975; Ando *et al.*, 1989; Margosiak *et al.*, 1990; Folk and Chung, 1973). Platelet and chicken gizzard transglutaminases have been reported to be activated by CaM (Puszkin and Raghuraman, 1985). Another report showed that CaM indirectly inhibited the activity of blood coagulating factor XIII (a plasma transglutaminase) by lowering the free Ca^{2+} concentration through chelation (Cohen *et al.*, 1986). Here, we report (a) the effect of CaM on the nucleotide hydrolysis activity which is a recently reported activity associated with guinea pig liver transglutaminase (Lee *et al.*, 1989, 1993), and (b) evidence for the direct binding of CaM to transglutaminase.

Abbreviations: CaM, calmodulin; dCaM, dansylated calmodulin; TBS, 40 mM Tris-HCl (pH 7.5)/150 mM NaCl.

Reprint requests to Dr. Y. Takeuchi, Banyu Tsukuba Research Institute, 3 Okubo, Technopark Oho, Tsukuba, Ibaraki 300-33, Japan.
Telefax: (0298) 77-2027.

Materials and Methods

Reagents

ATP, GTP, bovine brain CaM and its dansylated form (dCaM) were purchased from Sigma. The purity of CaM was confirmed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (Laemmli, 1970). Lyophilized CaM was reconstituted in TBS. Guinea pig liver transglutaminase was purified to homogeneity from a commercial source (Sigma) by GTP-agarose affinity chromatography, as previously reported (Lee *et al.*, 1989) except that 2.5 M KCl was used to elute the protein. The purified enzyme was stored in aliquots at −70 °C. Protein concentration were determined by Bradford method (Bradford, 1976).

0939–5075/94/0700–0453 $ 06.00

Enzyme assay

ATP and GTP hydrolysis activity was determined in the presence or absence of Ca^{2+} as previously reported (Lee *et al.*, 1989; Kikuchi *et al.*, 1988), using 2 μM [γ-^{32}P]ATP or [γ-^{32}P]GTP (New England Nuclear), respectively, in 100 μl TBS containing 5 mM $MgCl_2$. The transglutaminase activity was measured in 0.2 ml TBS as reported (Lorand *et al.*, 1972) using 1 mM [1,4-^{14}C]-putrescine (Amersham) and 0.5 mg N,N-dimethylated casein (Calbiochem). In the latter assay, $K_{pH^{Ca\text{-}EGTA}}$ value of 4.4×10^6 M^{-1} (Puszkin and Raghuraman, 1985) was applied to obtain the desired free Ca^{2+} concentration in the presence of 30 μM EGTA. CaM was present in each assay at the indicated concentrations. CaM itself did not show any significant activity as nucleotidase or transglutaminase, neither worked as a substrate for transglutaminase.

Fluorescence spectrophotometry

In order to detect an interaction between transglutaminase and CaM, 0.4 μM dCaM was incubated in 0.4 ml TBS at 0 °C with (a) 30 μM EDTA/EGTA as a control, (b) 0.4 μM (12.3 μg) transglutaminase plus 250 μM EDTA/EGTA, (c) 210 μM $CaCl_2$/30 μM EDTA/EGTA, or (d) 0.4 μM transglutaminase plus 210 μM $CaCl_2$/30 μM EDTA/EGTA. At the indicated times, the mixture was excited at 340 nm and the emission spectrum was obtained over the range of 400–600 nm (Kincaid *et al.*, 1982) using an Aminco-Bowman spectrofluorometer. Buffer alone, buffer containing $CaCl_2$, EDTA/EGTA, and/or transglutaminase gave no significant fluorescence in the wave-length range tested.

Results

CaM effect on enzymatic activities of transglutaminase

We previously reported GTP hydrolysis activity of guinea pig liver transglutaminase (Lee *et al.*, 1989). We found that ATP was similarly hydrolyzed by the enzyme as shown in Fig. 1. The effect of CaM on both ATP and GTP hydrolysis activities associated with guinea pig liver transglutaminase was tested. ATP hydrolysis was transiently enhanced and then gradually decreased depending on CaM concentration (Fig. 1 A and C). The biphasic effect of CaM was also observed in GTP hydrolysis activity, although to a lesser extent (Fig. 1 B and D). The maximal enhancement in hydrolytic activities was obtained at ≈0.1 μM CaM for 0.11 μM transglutaminase and at ≈0.2 μM CaM for 0.22 μM transglutaminase. Such a CaM effect on nucleotide hydrolysis activity was poorly dependent on the presence of Ca^{2+}. When the CaM effect on the classical transglutaminase activity was tested, it was small but the peak activity was observed again around an equimolar ratio of CaM to the enzyme (Fig. 2). We further tested Ca^{2+} dependency of CaM effect on the transglutaminase activity. However, it was only minor at a stoichiometric, or even excess, CaM:enzyme molar ratio (data not shown).

Fluorescence spectrophotometry

In order to demonstrate the interaction between transglutaminase and CaM, we employed dCaM whose fluorescence signal was revealed to change depending on specific CaM-binding protein (Kincaid *et al.*, 1982). The fluorescence spectrum of dCaM indeed altered significantly in the presence of transglutaminase, Ca^{2+} or both (Fig. 3 A). The emission peak shifted from 525–530 nm to (a) 517–520 nm with the addition of an equimolar amount of the enzyme, (b) 500–503 nm with Ca^{2+} and (c) 488–493 nm when both transglutaminase and Ca^{2+} were present. The fluorescence intensity of dCaM increased in the presence of the enzyme and/or Ca^{2+} (Fig. 3 A). The emission pattern of dCaM did not shift when BSA, instead of transglutaminase, was present (data not shown).

The time course of emission intensity of dCaM is illustrated in Fig. 3 B. The intensity decreased gradually most likely as a consequence of the denaturation of CaM molecule. However, the addition of Ca^{2+}, transglutaminase or both, all protected dCaM from the fluorescence diminution, though to differing extents (transglutaminase plus Ca^{2+} > transglutaminase > Ca^{2+}). The results shown in Fig. 3 indicate an interaction of guinea pig liver transglutaminase with CaM. It may be emphasized that such interaction was significant even in the absence of Ca^{2+}.

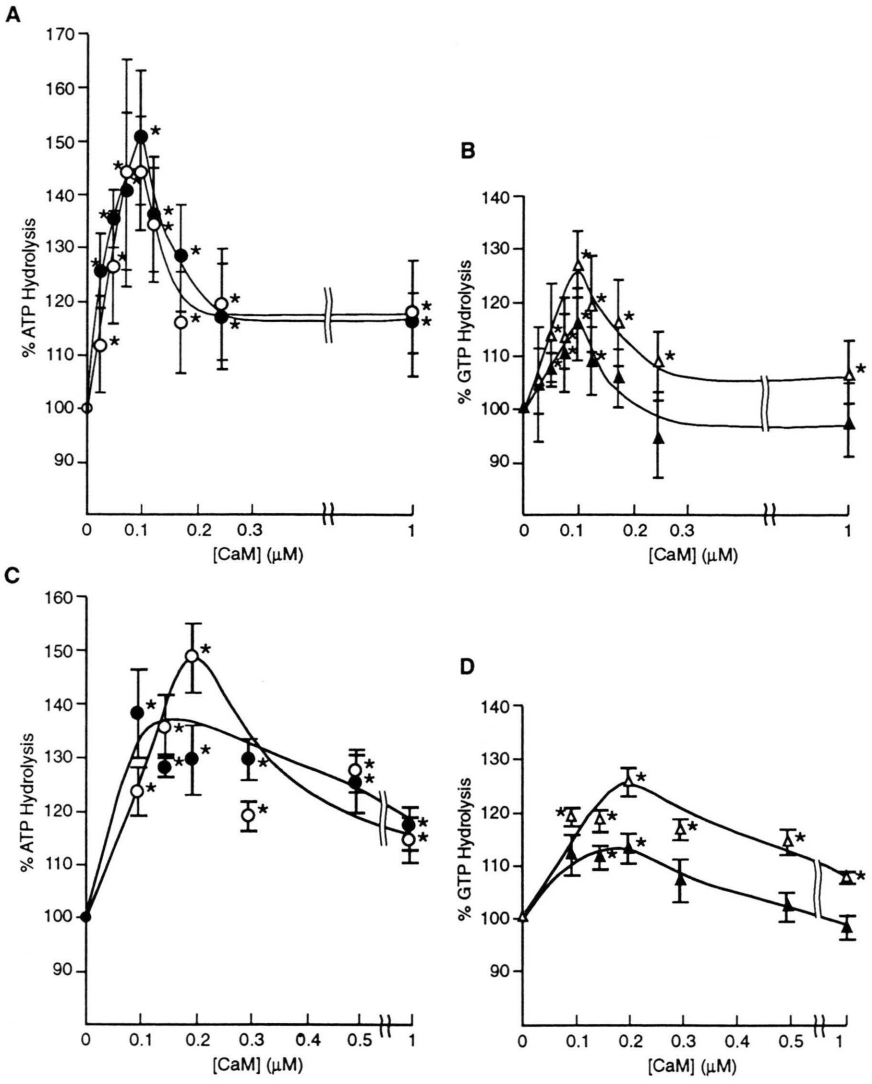

Fig. 1. Effect of CaM on the nucleotide hydrolysis activity of guinea pig liver transglutaminase. Purified transglutaminase in 100 μl was incubated for 30 min at 37°C as previously reported (Lee *et al.*, 1989) with the indicated concentrations of CaM plus 210 μM $CaCl_2$/30 μM EDTA/EGTA or 250 μM EDTA/EGTA. ^{32}Pi released by the enzyme action was calculated after subtracting the spontaneous release of ^{32}Pi from non-enzymatic sample. Enzyme amounts employed were 0.9 μg (0.11 μM, panels A and B) and 1.8 μg (0.22 μM, panels C and D), respectively. Panels A and C: ○, ATP/$+Ca^{2+}$; ●, ATP/$-Ca^{2+}$; panels B and D: △, GTP/$+Ca^{2+}$; ▲, GTP/$-Ca^{2+}$. Values represent mean ± SEM for replicate samples from 6–9 experiments. *: Significantly different ($p < 0.02$, student t-test) from no CaM sample. Control activities assayed in the absence of CaM were 12.40 ± 0.49, 13.52 ± 0.41, 10.64 ± 0.40 and 11.77 ± 0.52 nmol/mg·h for ATP/$+Ca^{2+}$, ATP/$-Ca^{2+}$, GTP/$+Ca^{2+}$ and GTP/$-Ca^{2+}$ samples, respectively.

Discussion

The dCaM fluorescence study revealed an interaction between transglutaminase and CaM (Fig. 3). Fig. 1 demonstrates regulation of the nucleotide hydrolysis activity of guinea pig liver transglutaminase by CaM. Interestingly, this CaM effect was biphasic. On the contrary to the reported CaM activation of platelet and chicken gizzard enzymes (Puszkin and Raghuraman, 1985), the CaM effect on the "classical" transglutaminase activity of guinea pig liver enzyme was small (Fig. 2). It is unlikely that the CaM effect was due to its Ca^{2+}-chelating ability, since (a) a large excess of Ca^{2+} to CaM was used in assay mixtures and (b) the biphasic CaM effect was also observed in the nucleotide hydrolysis assay from which Ca^{2+} was omitted (Fig. 1). Rather the biphasic CaM effect probably suggests that guinea pig liver transglutaminase contains multiple CaM-binding sites. As shown in Fig. 1 (and also in Fig. 2, though apparently less effective compared to Fig. 1), the maximal effect of CaM on the enzymatic activities of transglutaminase was observed around a stoichiometric ratio of the two proteins. It is also of interest to note that CaM site(s) of guinea pig liver

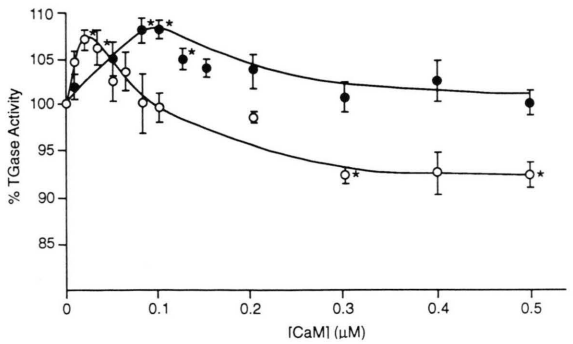

Fig. 2. Effect of CaM on the classical transglutaminase activity. In 200 μl, 0.53 μg (33 nM, —○—) or 1.6 μg (100 nM, —●—) of purified enzyme was assayed for transglutaminase activity in the presence of 60 μM $CaCl_2$ and 30 μM EGTA (thus, free $Ca^{2+} \approx 30$ μM) and indicated concentrations of CaM. Values represent mean ± SEM for replicate samples from 5 experiments. *: Significantly different ($p < 0.02$, student t-test) from no CaM sample. Control activity assayed in the absence of CaM was 6.41 ± 0.21 μmol/mg·h.

transglutaminase is essentially Ca^{2+}-independent as shown in Fig. 1 and 3. Further physicochemical and protein chemical studies are required to clarify the interaction between these two proteins. The physiological concentration of free Ca^{2+} in cells is at most 10 μM (usually ≈1 μM or lower), although Ca^{2+} concentration may oscillate during cellular processes (Berridge, 1990). In *in vitro* assay, the "classical" transglutaminase activity at 10 μM free Ca^{2+} was less than 10% of the full activity obtainable at >200 μM Ca^{2+}, and CaM showed only a small effect in a tested range (0.1–100 μM) of Ca^{2+} concentration (data not shown). Furthermore, GTP exists in a significant concentration to sufficiently suppress the tissue type transglutaminase activity (Achyuthan and Greenberg, 1987; Bergamini *et al.*, 1987). Thus, the tissue transglutaminase may not efficiently function as "classical" transglutaminase in a constant manner. To the contrary, the nucleotide hydrolysis activity of guinea pig liver enzyme is essentially Ca^{2+}-independent (Lee *et al.*, 1989 and Fig. 1). Hence, the enzyme may be more physiological as a nucleotidase rather than as a "classical" transglutaminase. Recent reports have proposed that tissue transglutaminase appears to be involved in various cellular processes that depend on growth factors and cytokines (Greenberg *et al.*, 1991; Piacentini *et al.*, 1991; Nara *et al.*, 1989; Kojima *et al.*, 1993; Fukuda *et al.*, 1993; Suto *et al.*, 1993). The fundamental role of tissue transglutaminase may be associated with a novel aspect of the enzyme as a nucleotidase.

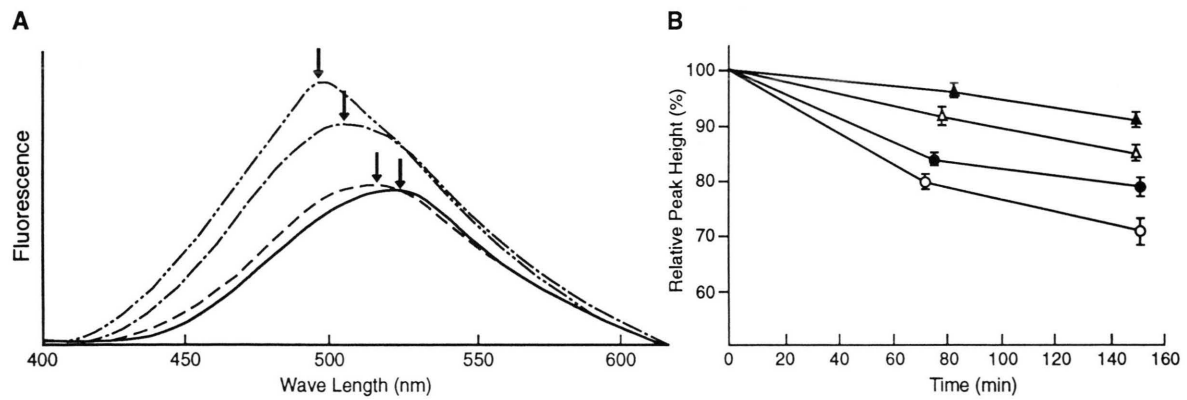

Fig. 3. Fluorescence spectrum of dCaM. A: Fluorescence spectroscopy of 0.4 μM dCaM was achieved as described in Materials and Methods in the presence of 250 μM EDTA/EGTA as a control (——); 0.4 μM transglutaminase plus 250 μM EDTA/EGTA (---); 210 μM $CaCl_2$/30 μM EDTA/EGTA (– · –); and 0.4 μM transglutaminase plus 210 μM $CaCl_2$/30 μM EDTA/EGTA (– · · –). Arrows indicate the emission peaks of each spectrum. B: Protection of dCaM by Ca^{2+} and transglutaminase from denaturation. Fluorescence spectrum of dCaM was produced at the indicated times. Peak height relative to 0 time peak was plotted. ○, control; ●, +$CaCl_2$; △, +transglutaminase; ▲, +$CaCl_2$ + transglutaminase. Values represent mean ± SEM from four separate experiments. No significant loss of enzymatic activities was observed during the assay period depicted in B.

Achyuthan K. E. and Greenberg C. S. (1987), Identification of a guanosine triphosphate binding site on guinea pig liver transglutaminase. Role of GTP and calcium ion in modulating activity. J. Biol. Chem. **262,** 1901–1906.

Ando H., Adachi M., Umeda K., Matsuura A., Nonaka M., Uchio R., Tanaka H. and Motoki M. (1989), Purification and characteristics of a novel transglutaminase derived from microorganisms. Agric. Biol. Chem. (Tokyo) **53,** 2613–2617.

Bergamini C. M., Signorini M. and Poltronieri L. (1987), Inhibition of erythrocyte transglutaminase by GTP. Biochim. Biophys. Acta **916,** 149–151.

Berridge M. J. (1990), Calcium oscillations. J. Biol. Chem. **265,** 9583–9586.

Bradford M. (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248–254.

Chung S. I. (1975), Isozymes **1** (C. L. Markert, ed.). Academic Press, New York, pp. 259–273.

Cohen I., Kahn D. R. and Drisdel R. C. (1986), Inhibition of platelet factor XIIIa-catalyzed reaction by calmodulin. Biochim. Biophys. Acta **883,** 265–270.

Folk J. E. and Chung S. I. (1973), Molecular and catalytic properties of transglutaminases. Adv. Enzymol. **38** (A. Meister, ed.). John Wiley & Sons, New York, pp. 109–191.

Folk J. E. and Finlayson J. S. (1977), The ε-(γ-glutamyl)-lysine cross-link and the catalytic role of transglutaminases. Adv. Protein Chem. **31,** 1–133.

Fukuda K., Kojiro M. and Chiu J.-F. (1993), Induction of apoptosis by transforming growth factor-β1 in the rat hepatoma cell line McA-RH 7777: A possible association with tissue transglutaminase expression. Hepatology **18,** 945–953.

Greenberg C. S., Birckbichler P. J. and Rice R. H. (1991), Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. FASEB J. **5,** 3071–3077.

Kikuchi A., Yamashita T., Kawata M., Yamamoto K., Ikeda K., Tanimoto T. and Takai Y. (1988), Purification and characterization of a novel GTP-binding protein with a molecular weight of 24,000 from bovine brain membranes. J. Biol. Chem. **263,** 2897–2904.

Kincaid R. L., Vaughn M., Osborne J. C., Jr. and Tkachuk V. A. (1982), Ca^{2+}-dependent interaction of 5-dimethylaminonaphthalene-1-sulfonyl calmodulin with cyclic nucleotide phosphodiesterase, calcineurin and troponin I. J. Biol. Chem. **257,** 10638–10643.

Klee C. B. and Cohen P. (1988), in: Molecular Aspects of Cell Regulation, **Vol. 5.** Elsevier Scientific Publishing Co., New York.

Kojima S., Nara K. and Rifkin D. B. (1993), Requirement for transglutaminase in the activation of latent transforming growth factor-β in bovine endothelial cells. J. Cell Biol. **121,** 439–448.

Laemmli U. K. (1970), Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685.

Lee K. N., Birckbichler P. J. and Patterson M. K., Jr. (1989), GTP hydrolysis by guinea pig liver transglutaminase. Biochem. Biophys. Res. Commun. **162,** 1370–1375.

Lee K. N., Arnold S. A., Birckbichler P. J., Patterson M. K., Jr., Fraij B. M., Takeuchi Y. and Carter H. A. (1993), Site-directed mutagenesis of human tissue transglutaminase: Cys-277 is essential for transglutaminase activity but not for GTPase activity. Biochim. Biophys. Acta **1202,** 1–6.

Lorand L., Campbell-Wilkes L. K. and Cooperstein L. (1972), A filter paper assay for transamidating enzymes using radioactive amine substrates. Anal. Biochem. **50,** 623–631.

Lorand L. and Conrad S. M. (1984), Transglutaminases. Mol. Cell. Biochem. **58,** 9–35.

Margosiak S. A., Dharma A., Bruce-Carver M. R., Gonzales A. P., Louie D. and Kuehn D. (1990), Identification of the large subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase as a substrate for transglutaminase in *Medicago sativa* L. Alfalfa. Plant Physiol. **92,** 88–96.

Nara K., Nakanishi K., Hagiwara H., Wakita K., Kojima S. and Hirose S. (1989), Retinol-induced morphological changes of cultured bovine endothelial cells are accompanied by a marked increase in transglutaminase. J. Biol. Chem. **264,** 19308–19312.

Piacentini M., Autori F., Dini L., Farrace M. G., Ghibelli L., Piredda L. and Fesus L. (1991), "Tissue" transglutaminase is specifically expressed in neonatal rat liver cells undergoing apoptosis upon epidermal growth factor stimulation. Cell Tissue Res. **263,** 227–235.

Puszkin E. G. and Raghuraman V. (1985), Catalytic properties of a calmodulin-regulated transglutaminase from human platelet and chicken gizzard. J. Biol. Chem. **260,** 16012–16020.

Suto N., Ikura K. and Sasaki R. (1993), Expression induced by interleukin-6 of tissue type transglutaminase in human hepatoblastoma HepG2 cells. J. Biol. Chem. **268,** 7469–7473.