Calreticulin Down-Regulates both GTP Binding and Transglutaminase Activities of Transglutaminase II[†]

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ABSTRACT: Enzyme regulation is an important mechanism for controlling cell proliferation and differentiation in response to extracellular signaling molecules. We have previously reported that a ∼50 kDa protein (termed Gβ_h) consistently copurified with Gα_h (transglutaminase II, TGII) and that Gβ_h down-regulates the GTPase function of TGII by associating with GDP-bound TGII [Baek et al. (1996) *Biochemistry 35*, 2651−2657]. In this study, we examined the identity of Gβ_h by partial amino acid sequencing and immunological characterizations. The results strongly suggest that Gβ_h is a protein known as calreticulin (CRT). When the regulatory role of CRT in the GTPase activity of TGII was examined, CRT inhibited GTP (GTPγS) binding and hydrolysis in a concentration-dependent manner. Moreover, CRT interacted only with GDP-bound TGII. These results demonstrate that CRT down-regulates the GTPase activity of TGII by associating with GDP-bound TGII. Studies on the modulation of the TGase activity of TGII revealed that CRT also inhibited TGase activity. The inhibition showed the two characteristics depend on guanine nucleotides occupying the GTPase active site. The inhibition of the "empty" form of the GTPase active site increased the Ca^{2+} requirement without changing the V_{max}. On the other hand, the inhibition of the GDP-bound form decreased V_{max}, but did not alter the Ca^{2+} requirement. Moreover, the GTPγS-bound TGII was virtually resistant to Ca^{2+}-mediated stimulation of the TGase activity, indicating that the GTP-bound TGII does not function as a TGase. We concluded that CRT is the regulatory protein of TGII that down-regulates both GTPase and TGase activities, opposing the activators of TGII function.

To date, all of the identified guanosine triphosphatases (GTPases) are known to undergo an activation/deactivation cycle. One of the well-characterized regulatory systems of GTPases is the α subunit (Gα) of the heterotrimeric GTPases (G-proteins),[1] which associates with the β and γ subunits (*1*, *2*). Activation of the heterotrimeric G-proteins is initiated by a receptor and involves several steps: stimulation of GDP release, which facilitates GTP binding, and dissociation of the $\beta\gamma$ subunits from GTP−Gα. The deactivation of Gα is initiated by the intrinsic GTPase reaction and terminated by the association of GDP-Gα with the $\beta\gamma$ subunits.

An unusual GTPase is a tissue-type transglutaminase (TGII or Gα_h) which exhibits two biological functions, GTPase and TGase (*R*-glutaminyl-peptide:amino-γ-glutamyltransferase) (*3*, *4*). The GTPase function of TGII appears to act as a signal mediator of hormone-receptor signaling systems, including the $\alpha_{1B/D}$-adrenoceptors (*4*−*6*) and the oxytocin receptor system (*7*). The activation/deactivation process of the GTPase function of TGII is similar to that of the heterotrimeric G-proteins, involving a receptor as the GTPase activator and a ∼50 kDa protein (Gβ_h) as the negative regulator (*8*, *9*). Thus, the receptor facilitates GDP release from the TGII−Gβ_h complex. Once GTP is bound to the complex, Gβ_h dissociates from GTP−TGII. However, the receptor may not be involved in this step, since Gβ_h does not physically contact the receptor (*8*). Down-regulation of GTPase function involves the intrinsic GTPase activity and reassociation of free Gβ_h with GDP−TGII. The activation/deactivation process of TGase activity involves Ca^{2+}, the positive regulator, and guanine nucleotides, the negative regulator (*3*, *10*). Recent studies by Lai et al. (*11*) have demonstrated that sphingosylphosphocholine up-regulates TGase activity, accompanying a decrease in Ca^{2+} requirement. These observations suggest that TGase activity is modulated by multiple factors.

Although the regulation of GTPase activity of TGII by Gβ_h has been studied (*8*), the regulation of TGase activity by this protein is not known. Moreover, the identity of Gβ_h is completely unknown. In this study, we have found that amino acid sequences of Gβ_h are identical to those of CRT. We, therefore, examined the regulatory role of CRT on both GTPase and TGase activities of TGII by immunological and

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[1] Abbreviations: CRT, calreticulin; TGII, tissue type transglutaminase which is also a GTPase; G-protein, GTP binding regulatory protein; GTP, guanosine 5′-triphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GDP, guanosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; AppNHp, adenyl-5′-yl imidodiphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DTT, 1,4-dithiothreitol.

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functional studies. Here, we provide data showing that CRT is the regulatory protein of TGII that down-regulates both GTPase and TGase functions. In addition, a role for the GDP-bound form of TGII in TGase function is suggested, and a plausible regulation mechanism of TGII by CRT is discussed.

EXPERIMENTAL PROCEDURES

Materials. Protein A−agarose, GTP−agarose, *N,N′*-dimethylated casein, and Stains All were purchased from Sigma. Nucleotides were from Boehringer Mannheim (Indianapolis, IN). Sucrose monolaurate (SM-1200) was obtained from Mitsubishi-Kasei Co. (Tokyo, Japan). Dimethyl pimelimidate dihydrochloride was purchased from Pierce (Rockford, IL), and [^{3}H]putrescine (37.5 Ci/mmol), chemiluminescence reagent, [^{35}S]GTPγS (∼1300 Ci/mmol), and [α-^{32}P]GTP (3000 Ci/mmol) were from Du Pont New England Nuclear (Boston, MA). Polyclonal antibodies against $G_{h7\alpha}$ (bovine TGII) and rat Gβ_h were raised in our laboratory (*8*, *12*), and a polyclonal antibody against calreticulin was from Affinity Bioreagents Inc. (Golden, CO). Monoclonal TGII (clone CUB 7402) was obtained from NeoMarkers (Fremont, CA). ProBlott membranes were purchased from Applied Biosystem (Foster, CA), and Mobilon-P membranes were from Millipore (Bedford, MA).

Purification of Proteins. A complex of TGII associated with Gβ_h (TGII−Gβ_h) was purified from rat liver (*9*, *12*). Rat liver TGII was purified using GTP−agarose with a slight modification of the method described by Achyuthan and Greenberg (*10*). Thus, the purified TGII−Gβ_h complex (∼100 nM) was incubated batchwise with GTP−agarose (3 mL) overnight at 4 °C. The resins were transferred to a column and intensively washed with HDGD buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT) containing 150 mM NaCl and 0.02% sucrose monolaurate (SM). The bound TGII was eluted with 1 M KCl in HDGD buffer containing 0.02% SM, collecting 500 μL fractions. The fractions containing pure TGII were selectively pooled and concentrated through a Centricon (Amicon Corp.). The concentrated samples were treated with ammonium sulfate (40% final) in an ice bath. The precipitates were collected and dissolved in HSDS buffer solution (20 mM HEPES, pH 7.4, 100 mM NaCl, 0.5 mM DTT, and 0.02% SM) and loaded onto a dry G-25 Sephadex column preequilibrated with the same buffer solution. This preparation was used as the empty form of the GTP binding site of TGII (E−TGII). The stability of E−TGII was less than 1 month in HSDS buffer solution containing 10% glycerol at −80 °C. Gβ_h was obtained using two different methods: (i) the flow-through and wash fractions obtained from the above GTP−agarose column were applied to a Mono Q-Sepharose column and eluted with a step gradient of NaCl (50−600 mM) in HDGD buffer containing 0.02% SM; (ii) the purified TGII−Gβ_h complex pretreated with 50 μM GTP and 2 mM $MgCl_2$ for 20 min at 30 °C was applied to a Mono Q-Sepharose column and eluted with a step gradient of NaCl (50−600 mM) in the same buffer (*8*). Throughout the purification, Gβ_h was monitored using Stains All (*13*) and immunoblotting with Gβ_h or CRT antibody. For the studies, the final Gβ_h preparation was subjected to N-terminal sequencing to determine the purity and to be sure that the protein was CRT.

For the coimmunoprecipitation studies, TGII and CRT were partially purified from rat liver membranes by one-step chromatography using Mono-Q Sepharose (*9*, *12*). Briefly, the membrane extract (1 g of protein) was preincubated with 50 μM GTP and 5 mM $MgCl_2$ at 30 °C for 20 min and loaded on the column which was preequilibrated with HDGD buffer containing 50 μM GTP, 2 mM $MgCl_2$, and 0.02% SM. The bound proteins were eluted using a linear gradient (50−600 mM of NaCl) in HDGD buffer containing 50 μM GTP, 5 mM $MgCl_2$, and 0.02% SM. The fractions were subjected to immunoblotting with antibodies specific to TGII and CRT (or Gβ_h) and selectively pooled to prevent TGII and CRT from contaminating each other. The resolved TGII and CRT were also monitored with Stains All (*13*). Excess salts were removed by a dry G-25 Sephadex column preequilibrated with HSDS buffer solution. Prior to use, the preparations were rechromatographed on a G-25 Sephadex column to remove the remaining guanine nucleotide. The amounts of TGII and CRT were estimated by quantitating the autoradiogram of immunoblots using a densitometer (Fotodyne Inc. Vari Quest 100 apparatus). Known concentrations of purified guinea pig liver TGII and Gβ_h were used as the standard.

Sequencing. The amino-terminal amino acid sequence of Gβ_h was obtained using the purified TGII−Gβ_h complex from rat liver. The Gβ_h was separated from TGII by SDS−PAGE (10% gel) and then transferred to a ProBlott membrane. After being stained with Coomassie blue, the Gβ_h band was subjected to N-terminal sequencing as presented in Table 1. To obtain internal sequences of Gβ_h, the purified Gβ_h was digested with CNBr in 0.1 N HCl (20 mg/mL) in the dark at room temperature for 24 h. Fragments were separated using a Beckman Gold Nouveou high-performance liquid chromatography (HPLC) system fitted with a reverse-phase C2/C18 column (sc 2.1 × 10 mm) from Amersham Pharmacia Biotech. The column was preequilibrated with the HPLC-grade water containing 0.085% trifluoroacetic acid. The bound peptides were eluted at a flow rate of 0.4 mL/min with a gradient of 90% CH_3CN in water in the following gradient: 5−60% over a period of 50 min followed by 60−90% for 10 min. A peptide fragment was then subjected to amino acid sequence analysis (Table 1). The sequencing was performed in the Biotechnology Core Facility of the Lerner Research Institute at The Cleveland Clinic Foundation.

Coimmunoprecipitation. For the coimmunoprecipitation studies, immunoaffinity resins of TGII, CRT, and Gβ_h antibodies were prepared (*8*). The mixture of partially purified TGII (∼3 nM) and CRT (∼3 nM) was incubated with various nucleotides, 2 mM $MgCl_2$, and the immunoaffinity resins (50 μL) overnight at 4 °C. After collecting by centrifugation (500 g), the resins were washed 3 times (1 mL/wash) with HSDS buffer solution containing 2 mM $MgCl_2$. Coimmunoprecipitation of the associated proteins was determined by immunoblotting with the appropriate antibody. Changes in methods are described in detail in the figure legends.

GTP Binding and Hydrolysis. The purified E−TGII and CRT were used. E−TGII (1 nM) was mixed with various concentrations (0−9 nM) of CRT in HSDS buffer solution. For photoaffinity labeling, the mixture of E−TGII and CRT was incubated with 20 μCi of [α-^{32}P]GTP in the presence of 2 mM $MgCl_2$ at room temperature for 20 min, transferred

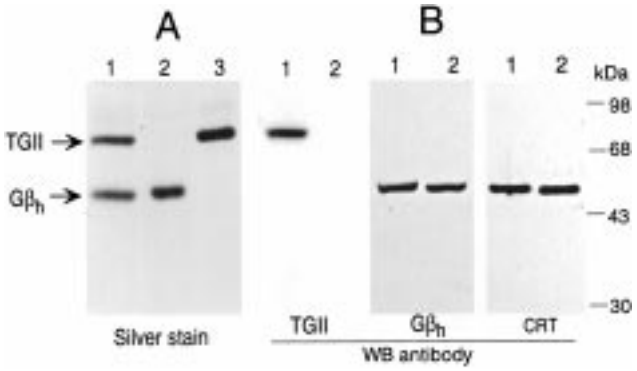

FIGURE 1: (A) Purity of the TGII−Gβ_h complex, Gβ_h, and E−TGII. The purified proteins were visualized by silver staining, following SDS−PAGE (11% gel). Lane 1, rat liver TGII−Gβ_h (1 nM GTPγS binding); lane 2, Gβ_h (2 nM estimated by immunoblotting); lane 3, purified rat liver TGII (1 nM estimated by immunoblotting). (B) Cross-reactivity of CRT antibody with Gβ_h in the TGII−Gβ_h complex and free Gβ_h. The same amounts of proteins shown in panel A were subjected to Western blotting (WB) using TGII or Gβ_h or CRT antibody. Proteins applied to the immunoblotting in lanes 1 and 2 are the same as those in lanes 1 and 2 in panel A.

to an ice-bath, and cross-linked for 8 min by UV irradiation (*9*). The bound radiolabeled GTP and GDP were visualized by autoradiography, following SDS−PAGE (10% gel). For GTP hydrolysis, the samples were incubated with 5 μM [α-^{32}P]GTP (specific activity 88 000 cpm/nM) and 2 mM $MgCl_2$ at 30 °C for 30 min in a 30 μL final volume. The reaction was stopped by adding an equal volume of 10% SDS. An aliquot (5 μL) of samples was spotted onto polyethyleneimine-cellulose-fluorescene plates (Merck) and run in a modified solvent system (0.75 M Tris-HCl−1 M LiCl/ethanol, 4:1) (*14*). The ^{32}P-labeled guanine nucleotides were visualized by autoradiography.

Other Experiments. GTPγS binding assay was performed using 2 μM [^{35}S]GTPγS (specific activity 3300 cpm/nM) (*9*). TGase activity was determined using 1 μCi of [^{3}H]-putrescine and 1% *N,N*-dimethylcasein (final) as the substrates (*10*). Protein concentrations were determined by the method of Bradford (*15*), using bovine serum albumin as a standard.

RESULTS

Identity of Gβ_h. Gβ_h was purified as the complex form of TGII−Gβ_h from rat liver and as a dissociated form from TGII as shown in Figure 1A. The identity of Gβ_h was determined by amino acid sequencing of the N-terminal and/or internal CNBr peptide fragments (Table 1). Sequencing data of the N-terminus of Gβ_h in the complex and of an internal CNBr peptide fragment revealed that the amino acid alignment of Gβ_h exactly matched that of the mature form of rat calreticulin from which 17 amino acid residues of signal peptide were removed (Table 1). The N-terminal sequencing of Gβ_h purified from human heart tissues also showed an exact match with the mature form of human CRT (data not shown). Further immunological characterization also revealed that the polyclonal antibody against CRT recognized Gβ_h in both the TGII−Gβ_h complex and the free Gβ_h preparations from rat liver (Figure 1B). These findings strongly suggest that Gβ_h is the mature form of calreticulin. However, Gβ_h, being one of the calreticulin isoforms, or the related proteins, is not excluded, since amino acid sequences

Table 1: Results of N-Terminal Sequence Analysis of Gβ_h and Amino Acid Sequencing of a Peptide Obtained after Cleavage of Gβ_h with CNBr[a]

N-terminal sequence				CNBr fragment sequence			
cycle	aa residue	cycle	aa residue	cycle	aa residue	cycle	aa residue
1	D(29.42)	14	D(21.79)	1	K(10.98)	14	E(5.52)
2	P(59.43)	15	A(82.52)	2	D(6.41)	15	E(5.65)
3	A(63.33)			3	K(8.36)	16	D(3.16)
4	I(45.40)			4	Q(6.95)	17	K(3.27)
5	Y(39.83)			5	D(6.05)	18	K(5.41)
6	F(62.81)			6	E(5.10)	19	R(5.99)
7	K(55.64)			7	E(6.81)	20	K(4.43)
8	E(28.76)			8	Q(5.67)	21	E(2.81)
9	Q(20.76)			9	R(8.07)	22	E(3.67)
10	F(53.59)			10	L(5.62)	23	E(4.05)
11	L(54.68)			11	K(5.60)	24	E(4.04)
12	D(16.43)			12	E(4.29)	25	A(2.33)
13	G(18.47)			13	E(5.43)		

[a] Amino acids (aa) were detected as phenylthiohydantoin (PTH)-derivatives by Edman degradation in an automated protein sequencer. The single-letter code of the amino acid is given with the amount of PTH-derivative recovered indicated in parentheses (in picomoles). The CNBr cleavage occurred precisely at a methionine residue at position 360 in the rat calreticulin sequence. The regions of the amino acid sequences of Gβ_h were ^{18}D−^{32}A and ^{358}K−^{382}A of rat calreticulin (Gene Bank accession number P18418).

of Gβ_h are partial and the cross-reactivity of an antibody depends on the epitope regions of a protein.

Regulation of GTPase Function of TGII by CRT. The regulatory role of Gβ_h in the GTPase function of TGII encompasses two characteristics: Gβ_h (i) induces the inactive conformation of TGII and (ii) associates with GDP−TGII, thus inhibiting GTP binding to TGII (*8*). To investigate whether CRT is able to inhibit GTP binding to TGII, E−TGII was reconstituted with various concentrations of CRT in a neutral detergent solution. Effects of CRT on GTP binding to E−TGII were assessed by photoaffinity labeling and GTP hydrolysis with [α-^{32}P]GTP. The level of covalently bound [α-^{32}P]GTP (and GDP) to TGII decreased in a CRT dose-dependent manner (Figure 2A). Similarly, hydrolysis of [α-^{32}P]GTP by TGII was also inhibited (Figure 2B). Inhibition of GTPγS binding to TGII was also observed (Figure 2C). Based on the results of both GTP hydrolysis and GTPγS binding, the rate of GTP hydrolysis by TGII was estimated. The results showed that turnover of GTP hydrolysis (1−3 mol mol^{-1} min^{-1}) was not changed, demonstrating that inhibition of GTP hydrolysis by CRT is due to the inhibition of GTP binding to TGII. Since GTP binding to E−TGII is inhibited by CRT, these results also indicated that CRT induces TGII into an inactive conformation.

Next, we evaluated whether CRT interacts with GDP−TGII as Gβ_h did. To elucidate specific interactions between TGII and CRT, crude preparations of both proteins were mixed and incubated with various guanine nucleotides in the presence of $MgCl_2$. Since it has been demonstrated that TGII binds ATP and hydrolyzes it (*16*, *17*), adenine nucleotides were also included. Nucleotide effects on the interaction between these two proteins were determined by coimmunoprecipitation using the Gβ_h, CRT, and TGII antibody affinity resins (Figure 3). The control Gβ_h antibody coimmunoprecipitated a substantially higher level of TGII in the presence of GDP than in the presence of other nucleotides or absence of nucleotide. Similar results were obtained with the CRT antibody. Furthermore, the TGII antibody also coimmuno-

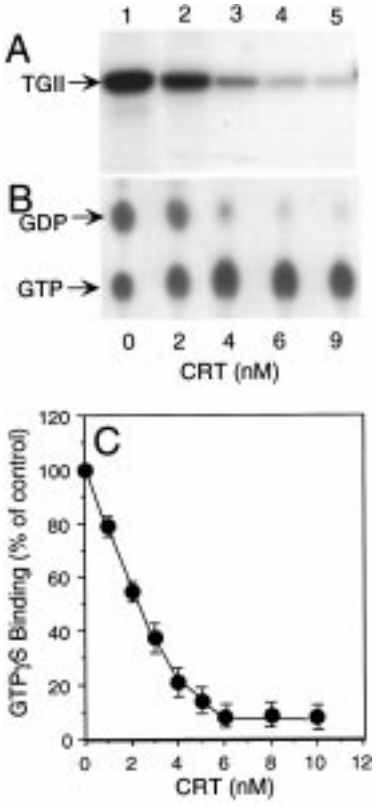

FIGURE 2: Effects of calreticulin on GTPase function of E−TGII. The purified E−TGII (1 nM) was reconstituted with various concentrations of the purified CRT as indicated in the figure. (A) Inhibition of GTP binding to TGII by CRT. The cross-linking of the bound radiolabeled GTP to TGII was visualized by autoradiography, following SDS−PAGE (10% gel). (B) Effects of CRT on GTP hydrolysis by TGII. The GDP produced was separated from GTP by thin-layer chromatography and visualized by autoradiography. (C) Inhibition of GTPγS binding to TGII by CRT. A constant amount of E−TGII (1 nM) was mixed with various concentrations of CRT in HSDS buffer solution. The mixtures were incubated with 2 μM [^{35}S]GTPγS (specific activity, 3300 cpm/nM) and 2 mM $MgCl_2$ at 30 °C for 30 min in a 100 μL final volume. Nonspecific binding was determined in the presence of 0.5 mM GTP. The bound GTPγS was trapped with nitrocellulose filters by fast filtration. The sample without CRT was taken as the control (100%). The results presented are means of two duplicate experiments.

precipitated more CRT in the presence of GDP than in the presence of other nucleotides. These results clearly indicate that CRT interacts with GDP−TGII. In the absence of nucleotide (Figure 3), failure of the coimmunoprecipitation of the associated proteins is most likely due to a weak interaction between CRT and E−TGII, since CRT was able to inhibit GTP (and GTPγS) binding to E−TGII (Figure 2A,C). These results suggest that the conformation of E−TGII differs from that of the GDP-bound form and is probably similar to that of the adenine nucleotide-bound form. Such conformational changes of TGII by nucleotides have been observed by others (*18*). Thus, GTP-bound fusion TGII protein was resistant to tryptic digestion, while the ATP-bound form was sensitive to tryptic digestion, exhibiting a similar pattern of fragmentation to TGII in the absence of nucleotide.

Regulation of TGase Function of TGII by Nucleotides. It is now well established that TGII possesses two biological enzyme activities, GTPase and TGase, that have been shown to be modulated by the counterpart inhibitors Ca^{2+} and GTP

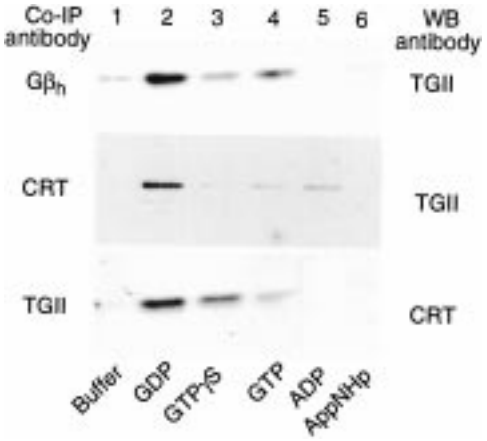

FIGURE 3: Interaction of CRT with TGII in the presence of various nucleotides. Equal amounts (~3 nM) of the resolved TGII and CRT were reconstituted in HSDS buffer solution and preincubated with various nucleotides at 30 °C for 20 min in a 500 μL final volume. Concentrations of nucleotides were 200 μM GDP, 10 μM GTPγS, 50 μM GTP, 300 μM ADP, and 300 μM AppNHp. Coimmunoprecipitation (Co-IP) of TGII or CRT was determined by Western blotting (WB) using the corresponding antibodies as indicated in the figure. The volume of the immunoaffinity resins was 50 μL (packed volume). (A) Coimmunoprecipitation of TGII with Gβ_h antibody. (B) Coimmunoprecipitation of TGII with CRT antibody. (C) Coimmunoprecipitation of CRT with TGII antibody.

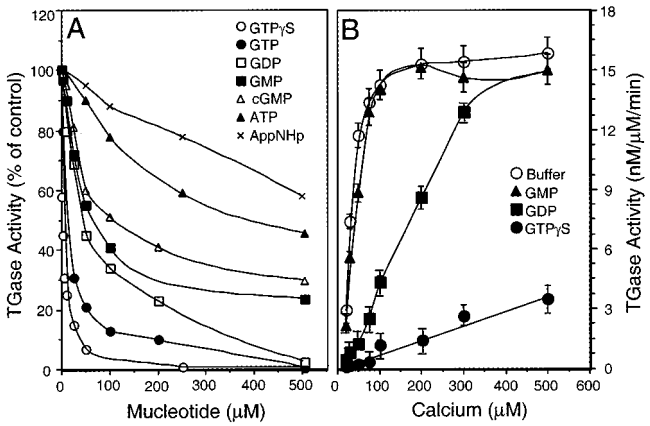

FIGURE 4: Modulation of TGase activity of TGII by nucleotides and Ca^{2+}. (A) Effects of nucleotides on Ca^{2+}-stimulated TGase activity of TGII. E−TGII (1 nM) was incubated with various concentrations of nucleotides in the presence of 100 μM Ca^{2+}, 2 mM $MgCl_2$, and the substrates at 30 °C for 30 min. Nonspecific TGase activity was determined in the absence of Ca^{2+} under the same conditions. The TGase activity of E−TGII without nucleotides was taken as the control (100%). Standard errors were ~10−17%. (B) Ca^{2+} effect on the guanine nucleotide-bound TGII. E−TGII was preincubated with GMP (300 μM), GDP (200 μM), and GTPγS (10 μM) in the presence of 2 mM $MgCl_2$. The TGase activity was determined with various Ca^{2+} concentrations at 30 °C for 30 min in a 100 μL final volume. The data shown in panels A and B are the means of two duplicate experiments.

(*10*, *19*, *20*). However, regulation of the TGase activity and the species of TGII among the nucleotide-unbound, GDP-bound, and GTP-bound forms which serve as TGase is not clearly understood. Therefore, the effects of various nucleotides on E−TGII were evaluated to determine whether Ca^{2+}-mediated TGase stimulation is affected (Figure 4A). Guanine nucleotides were potent inhibitors for Ca^{2+}-mediated TGase stimulation whereas adenine nucleotides showed a modest inhibitory effect, consistent with previous findings (*18*). The inhibitory potency of nucleotides was in the following

order: GTPγS > GTP > GDP ≫ GMP ≫ ATP > AppNHp. This order appears to be similar to that of the binding affinity of TGII for nucleotides (*9*). The effect of Ca^{2+} was then examined to evaluate whether Ca^{2+} reverses the guanine nucleotide-mediated TGase inhibition. In these experiments, TGII was preincubated with various guanine nucleotides, and then TGase activity was determined with varying Ca^{2+} concentrations (Figure 4B). The TGase activity of GMP−TGII and E−TGII was increased by Ca^{2+} in a dose-dependent manner and reached a plateau at ≥100 μM Ca^{2+}. Similarly, the enzyme activity of GDP−TGII increased in response to Ca^{2+} and also reached the level of E−TGII at ≥350 μM Ca^{2+}. The TGase activity of GTPγS−TGII increased slightly in response to Ca^{2+} and was about 4.7-fold less than other forms of TGII at 500 μM Ca^{2+} without reaching the plateau. These results indicate that GTPγS−TGII is resistant to Ca^{2+}-mediated TGase stimulation and that Ca^{2+} does not stimulate release of GTP (or GDP) from TGII once the guanine nucleotides are bound.

Regulation of TGase Function of TGII by CRT. Considering the findings that TGII hydrolyzes GTP to GDP and P_i and that association of CRT with GDP−TGII is the ground state of GTPase function, the regulatory role of CRT in the TGase function of TGII was evaluated with GDP−TGII and E−TGII (Figure 5). CRT inhibited the Ca^{2+}-mediated TGase stimulation of E−TGII, shifting the Ca^{2+} requirement to higher concentrations (Figure 5A). The shift in the Ca^{2+} requirement was CRT dose-dependent, increasing 2−3-fold over the requirement with E−TGII alone. The increased Ca^{2+} requirement can be due to the uptake of Ca^{2+} by CRT as CRT has high Ca^{2+} binding capacity (20−30 mol mol^{-1}) (*21*). However, Ca^{2+} concentrations in the reaction mixtures were much higher (2−6 nM CRT vs 200−300 μM Ca^{2+}) than the calcium binding capacity of CRT. Therefore, another mechanism is probably involved, as seen in inhibition of GTP binding to TGII by CRT; e.g., CRT induces an inactive conformation of TGII (Figure 2A,C). Furthermore, when the effects of CRT on TGase activity were evaluated with GDP−TGII, a gradual decrease in the V_{max} of the enzyme activity was observed by increasing CRT concentrations, but the Ca^{2+} requirement did not change (Figure 5B). These results strongly indicate that the inhibition of TGase activity of TGII is caused by allosteric hindrance of CRT, but not by the inhibition of substrate or Ca^{2+} binding to TGII. An additional experiment was performed to examine whether Ca^{2+} is involved in dissociation of CRT from the GDP−TGII−CRT complex. Coimmunopreciptation of TGII in the presence and absence of Ca^{2+} was performed with the Gβ_h and CRT antibodies (Figure 5C). The levels of TGII coimmunoprecipitated by both antibodies did not change, indicating that Ca^{2+} does not dissociate CRT from the complex. These findings clearly indicate that CRT is involved in down-regulation of TGase function and that the inhibition of TGase activity by CRT is not due to the inhibition of Ca^{2+} binding to TGII.

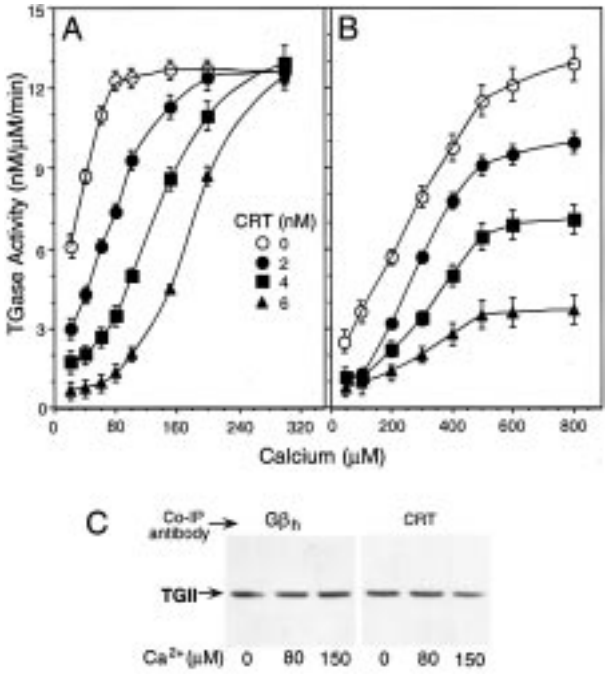

FIGURE 5: Effects of CRT on the TGase activity of E−TGII or GDP-TGII. (A) CRT effect on TGase activity of E−TGII in response to Ca^{2+}. E−TGII (1 nM) was reconstituted with various concentrations of CRT in HSDS buffer solution. The TGase activity was determined in the presence of various concentrations of Ca^{2+}. The concentrations of CRT reconstituted with TGII are shown as the inset in panel A. The results shown in panels A and B are means of two duplicate experiments. (B) CRT effect on the GDP-bound form of TGII in response to Ca^{2+}. The reconstitution of TGII with CRT was performed using the same conditions as described in panel A. The concentrations of CRT are as shown in the inset in panel A. The samples were preincubated with 300 μM GDP in the presence of 2 mM $MgCl_2$ at 30 °C for 20 min, and then the TGase activity was determined with varying Ca^{2+} concentrations at 30 °C for 30 min. (C) Ca^{2+} effect on interaction of CRT with GDP−TGII. The resolved TGII (3 nM) and Gβ_h (3 nM) preparations were mixed and incubated in the presence of 200 μM GDP and 2 mM $MgCl_2$ at 30 °C for 20 min in a 500 μL final volume. The samples were further incubated with various concentrations of Ca^{2+} with 50 μL of immunoaffinity resins of Gβ_h or CRT antibody overnight at 4 °C. Coimmunoprecipitation of TGII was determined by immunoblotting with TGII antibody.

DISCUSSION

We have previously reported that the GTPase function of TGII is down-regulated by a 50 kDa protein, Gβ_h (*8*). In the present study, we have described the findings that Gβ_h is most likely to be the protein previously known as calreticulin, and that this protein down-regulates both the GTP binding and TGase activities of TGII. The N-terminal and an internal peptide amino acid sequence of Gβ_h are identical to those of CRT (Table 1), and CRT antibody cross-reacts with Gβ_h, which copurifies with TGII (Figure 1B). Our studies on regulation of TGII functions revealed that CRT down-regulates both GTPase and TGase functions of TGII by association with GDP−TGII, and that GDP−TGII−CRT is the ground-state complex. Thus, CRT interacts only with the GDP-bound form of TGII that is not inhibited by Ca^{2+} (Figures 3 and 5C), consistent with previous findings with Gβ_h (*8*). Evidence that CRT down-regulates the TGase activity of TGII is provided by the observation that CRT inhibits TGase activity by altering the Ca^{2+} requirement for E−TGII and the V_{max} for GDP−TGII (Figure 5A,B). These data also suggest that CRT induces TGII to an inactive conformation, since the GTPase and TGase activities of E−TGII are able to be down-regulated by CRT.

To date, it is well established that regulation of the TGase activity of TGII requires Ca^{2+} and guanine nucleotide (*19*, *20*). However, it is poorly understood which guanine nucleotide-bound form of TGII serves as TGase in cells. Our

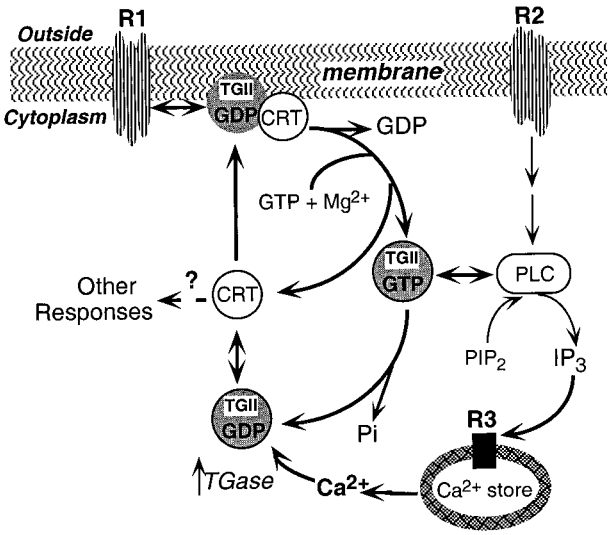

FIGURE 6: Schematic presentation of regulation of GTPase and TGase functions of TGII by CRT. The gray circle represents TGII. The Ca^{2+} store represents endoplasmic and/or sarcoplasmic reticulum. Abbreviations: R1, TGII activating receptors; R2, Ca^{2+}-mobilizing receptors; R3, IP_3 receptor; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-triphosphate.

results showed that the GTPγS−TGII is virtually resistant to stimulation by Ca^{2+} (Figure 4B), indicating that GTP−TGII may not function as a TGase. The other species (GMP−, GDP−, and E−TGII) were able to respond to Ca^{2+} in a dose-dependent manner (Figure 4B). Since GMP is not the natural ligand of TGII, E− or GDP−TGII is most likely to be the cellular species of the enzyme. Considering the findings that TGII, being a GTPase, has high affinity for GTP and hydrolyzes GTP to GDP and P_i (Figure 2A,B; *8*, *9*), it is unlikely that E−TGII exists in the cells. Supporting this idea, recent studies by Zhang et al. (*22*) have indicated that TGII which functions as TGase is a guanine nucleotide-bound form. Thus, depletion of GTP in human neuroblastoma SH-SY5Y cells resulted in an increase in basal TGase activity that may be due to E−TGII. On the other hand, induction of TGII by treating the cells with retinoic acid did not enhance the basal TGase activity that was stimulated by increasing Ca^{2+} concentrations. These responses are similar to the responses of GDP−TGII to Ca^{2+} (Figure 4B). Taken together, the observations by Zhang et al. (*22*) and our present studies show that the GDP-bound form of TGII is the likely candidate which functions as TGase.

Based on these and earlier findings (*8*−*10*, *18*, *22*), a plausible sequential activation/deactivation mechanism of GTPase and TGase functions of TGII is postulated (Figure 6). The relevant findings are the following: (i) TGII is a bifunctional enzyme having GTPase and TGase activities; (ii) both GTPase and TGase activities are down-regulated by CRT; (iii) CRT interacts with the GDP-bound form that is not inhibited by Ca^{2+}; (iv) CRT dissociates from GTP−TGII; (v) GTP−TGII is not stimulated by Ca^{2+}, but the GDP-bound form is; (vi) Ca^{2+} is the activating factor of TGase and up-regulates by external stimuli. Zhang et al. (*22*) also reported that activation of muscarinic receptor resulted in an increase in cross-linking of the TGase substrates in cells.

Calreticulin is a calcium binding protein with a high Ca^{2+} binding capacity and plays an important role in Ca^{2+} storage in endoplasmic/sarcoplasmic reticulum (*21*). Recent studies have shown that calreticulin is also found outside of these calcium store compartments. For example, immunoreactive calreticulin has been detected in the nuclear envelope and the nucleus of certain cell types (*23*), in the acrosome of sperm cells (*24*), and in the cytotoxic granules of T-cells (*25*). There is also evidence that the protein exists in the cytoplasm (*26*, *27*) and serum (*28*). Structural analysis of calreticulin has shown that the protein contains two calcium binding domains, P (proline rich)- and C (C-terminal)-domains (*29*). The P-domain is the high-affinity Ca^{2+} binding site with a low capacity (K_d, ~10 μM; binding capacity, 1 mol mol^{-1}), and the C-domain is the low-affinity Ca^{2+} binding site with a high capacity (K_d, ~250 μM; binding capacity, ~25 mol mol^{-1}). The high Ca^{2+} binding capacity of calreticulin is involved in Ca^{2+} storage. The finding of a high calcium binding site has been suggested to play a regulatory role (*21*, *29*). A large number of studies have demonstrated that calreticulin plays a regulatory role in signaling propagation of integrins and nuclear receptors (*30*−*35*). This regulatory role of calreticulin appears to be displayed in different ways depending on its cellular localization. Cytoplasmic calreticulin acts as the positive regulator by direct interaction with the cytoplasmic tail of the α subunits of integrins (*30*). On the other hand, the nuclear calreticulin functions as the negative regulator by direct interaction with a conserved region in the DNA binding domain of a variety of nuclear receptors, including retinoid receptor (*31*) and glucocorticoid receptor (*32*, 33). In the regulation involving the receptors, the effects of Ca^{2+} binding by clareticulin remain unclear. Our studies, however, indicate that in the regulation of TGII functions by CRT, Ca^{2+} binding to CRT may not affect the interaction of both proteins and the regulation of both GTPase and TGase activities. The interaction of CRT with GDP−TGII occurred without addition of Ca^{2+} (Figures 2 and 3), but also increasing Ca^{2+} concentrations did not cause the dissociation of CRT from the GDP−TGII−CRT complex (Figure 5C). The TGase activity of GDP−TGII was allosterically inhibited by CRT (Figure 5B), and uptake of Ca^{2+} by CRT was a minor factor in the increase in Ca^{2+} requirement to reverse the CRT-mediated TGase inhibition (Figure 5A). These observations suggest that induction of TGII to an inactive conformation by CRT is the primary regulatory mechanism in this system. Induction of a conformational change by CRT has been observed in the integrin system (*35*). The interaction of CRT with integrin induced the active state of the integrin which leads to adherence of the cells.

We have shown for the first time that CRT associates with TGII and regulates both GTPase and TGase functions. The findings are important for the better understanding of the mutifunctional properties of TGII and of calreticulin. The multifunctional roles of calreticulin suggest that this protein may belong to the calcium binding proteins that are involved in regulation of other proteins. For example, calmodulin, one of the well-characterized calcium binding proteins, has shown to regulate many enzymes, including adenylyl cyclase (*36*), nitric oxide synthase (*37*), and kinases (*38*, *39*). In addition, TGII has been shown to associate with a variety of cellular events, including cell adhesion (*40*−*42*), cell growth and proliferation (*43*, *44*), tumorigenesis (*45*−*47*), and heart failure (*48*, *49*). Although there is no evidence that TGII is involved in either integrin or retinoid receptor signaling, CRT

is also involved in regulating cell growth and differentiation, and cell adhesion (*21*). Therefore, it would be interesting to study whether TGII is involved in these CRT-mediated signaling processes.

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REFERENCES

1. Birnbaumer, L. (1990) *Annu. Rev. Pharmacol. Toxicol. 30*, 675−705.
2. Gilman, A. G. (1987) *Annu. Rev. Biochem. 56*, 615−649.
3. Im, M.-J., Russell, M. A., and Feng, J.-F. (1997) *Cell Signalling 9*. 477−482.
4. Nakaoka, H., Perez, D. M., Baek, K. J., Das, T., Husain, A., Misono, K., Im, M.-J., and Graham, R. M. (1994) *Science 264*, 1593−1596.
5. Im, M.-J. and Graham, R. M. (1990) *J. Biol. Chem. 265*, 18944−18951.
6. Chen, S., Lin, F., Iismaa, L. K. N., Birckbichler, P. J., and Graham, R. M. (1996) *J. Biol. Chem. 271*, 32385−32391.
7. Baek, K. J., Kwon, N. S., Lee, H. S., Kim, M. S., Murlaidhar, P., and Im, M.-J. (1996) *Biochem. J. 315*, 739−744.
8. Baek, K. J., Das, T., Gray, C. D., Desai, S., Hwang, K. C., Gacchui, R., Ludwig, M., and Im, M.-J. (1996) *Biochemistry 35*, 2651−2657.
9. Im, M. J., Riek, P. R., and Graham, R. M (1990) *J. Biol. Chem. 265*, 18952−18960.
10. Achyuthan, K. E., and Greenberg, C. S. (1987) *J. Biol. Chem. 262*, 1901−1906.
11. Lai, T.-S., Bielawska, A., Peoples, K. A., Hannun, Y. A., and Greenberg, C. S. (1997) *J. Biol. Chem. 272*, 16295−16300.
12. Baek, K. J., Das, T., Gray, C., Antar, S., Murugesan, G., and Im, M.-J. (1993) *J. Biol. Chem. 268*, 27390−27397.
13. Campbell, K. P., MacLennan, D. H., and Jorgensen, A. O. (1983) *J. Biol. Chem. 258*, 11267−11273.
14. DiBattiste, D., Goluble, M., Stacy, D., and Wolfman A. (1993) *Oncogene 8*, 637−643.
15. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
16. Lai, T. S., Slaughter, T. F., Koropchak, C. M., Haroon, Z. A., and Greenberg, C. S. (1996) *J. Biol. Chem. 273*, 31191−31195.
17. Iismaa, S. E., Chung, L., Wu, M.-J., Teller, D. C., Yee, V. C., and Graham, R. M. (1997) *Biochemistry 36*, 11655−11664.
18. Lai, T. S., Slaughter, T. F., Peoples, K. A., Hettasch, J. M., and Greenberg, C. S. (1998) *J. Biol. Chem. 273*, 1776−1781.
19. Greenberg, C. S., Birckbichler, P. J., and Rice, R. H. (1991) *FASEB J. 5*, 3071−3077.
20. Aeschlimann, D., and Paulsson, M. (1994) *Thromb. Haemostasis 71*, 402−415.
21. Nash, P. D., Opas, M., and Michalak, M (1994) *Mol. Cell. Biochem. 135*, 71−78.
22. Zhang, J., Lesort, M., Guttmann, R. P., and Johnson, G. V. W. (1998) *J. Biol. Chem. 273*, 2288−2295.
23. Opas, M., Dziak, E., Fliegel L., and Michalak, M. (1991) *J. Cell. Physiol. 149*, 160−171.
24. Nakamura, M., Moriya, M., Baba, T., Michikawa, Y., Yamanobe, T., and Arai, K. (1993) *Exp. Cell Res. 205*, 101−110.
25. Dupuis, M., Schaerer, E., Krause, K. H., and Tschopp, J. (1993) *J. Exp. Med. 177*, 1−7.
26. Rojiani, M. V., Finlay, B. B., Gray, V., and Dedhar, S. (1991) *Biochemistry 30*, 9859−9866.
27. McDonnell, J., Jones, G. E., White, T. K., and Tanzer, M. L. (1996) *J. Biol. Chem. 271*, 7891−7894.
28. Sueyoshi, T., McMullen, B. A., Marnell, L. L., Clos, T. W. D., and Kisiel, W. (1991) *Thromb. Res. 63*, 569−575.
29. Baksh, S., and Michalak, M. (1991) *J. Biol. Chem. 266*, 21458−21465.
30. Coppolino, M. G., Woodside, M. J., Demaurex, N., Grinstein, S., St-Arnaud, R., and Dedhar, S. (1997) *Nature 386*, 843−847.
31. Leung-Hagesteijn, C. Y., Milankov, K., Michalak, M., Wilkins, J., and Dedhar, S. (1994) *J. Cell Sci. 107*, 589−600.
32. Dedhar, S., Rennie, P. S., Shago, M., Leung-Hagesteijn, C.-Y., Yang, H., Filmus, J., Hawley, R. G., Bruchovsky, N., Cheng, H., Matusik, R. J., and Gigurere, V. (1994) *Nature 367*, 480−483.
33. St-Arnaud, R., Prudhomme, J., Leung-Hagesteijn, C., and Dedhar, S. (1995) *J. Cell Biol. 131*, 1351−1359.
34. Burns, K., Duggan, B., Atkinson, E. A., Famulski, K. S., Nemer, M., Bleackley, R. C., and Michalak, M. (1994) *Nature 367*, 476−480.
35. Coppolino, M., Leung-Hagesteijn, C.-Y., Dedhar, S., and Wilkins, J. (1995) *J. Biol. Chem. 270*, 23132−23138.
36. Sunahara, R. K., Dessauer, C. W., and Gilman, A. G. (1996) *Annu. Rev. Phamacol. Toxicol. 36*, 461−480.
37. Stuehr, D. J., Abu-Soud, H. M., Rousseau, D. L., Feldman, P. L., and Wang, J. (1995) *Adv. Pharmacol. 34*, 207−213.
38. Wei, J., Wayman, G., and Storm, D. R. (1996) *J. Biol. Chem. 271*, 24231−24235.
39. Piomelli, D., and Greengard, P. (1991) *Proc. Natl. Acd. Sci. U.S.A. 88*, 6770−6774.
40. Nara, K., Nakanishi, K., Hagiwara, H., Wakita, K., Kojima, S., and Hirose, S. (1989) *J. Biol. Chem. 264*, 19308−19312.
41. Jones, R. A., Nicholas, B., Mian, S., Davies, P. J. A., and Griffin, M. (1997) *J. Cell Sci. 110*, 2461−2472.
42. Gentile, V., Thomazy, V., Piacentini, M., Fesus, L., and Davies, P. J. A. (1992) *J. Cell Biol. 119*, 463−474.
43. Melino, G., Annicchiarico-Petruzzelli, M., Piredda, L., Candi, E., Gentile, V., Davies, P. J. A., and Piacentini, M. (1994) *Mol. Cell. Biol. 14*, 6584−6596.
44. Nagy, L., Thomazy, V. A., Saydak, M. M., Stein, J. P., and Davies, P. J. A. (1997) *Cell Death Differ. 4*, 534−547.
45. Hettasch, J. M., Bandarenko, N., Burchette, J. L., Lai, T.-S., Marks, J. R., Haroon, Z. A., Peters, K., Dewhirst, M. W., Iglehart, J. D., and Greenberg, C. S. (1996) *Lab. Invest. 75*, 637−645.
46. Birckbichler, P. J., Orr, G. R., Patterson, M. K., Jr., Conway, E., and Carter, H. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5005−5006.
47. Barnes, R. N., Bungay, P. J., Elliott, B. M., Walton, P. L., and Griffin M. (1985) *Carcinogenesis 6*, 459−463.
48. Hwang, K. C., Gray, C. D., Sweet, W. E., Moravec, C. S., and Im, M.-J. (1996) *Circulation 94*, 718−726.
49. Iwai, N., Shimoike, H., and Kinoshita, M. (1995) *Biochem. Biophys. Res. Commun. 209*, 527−534.

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