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Tissue Transglutaminase Is an In Situ Substrate of Calpain: Regulation of Activity

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Abstract: Tissue transglutaminase (tTG) is a calcium-dependent enzyme that catalyzes the transamidation of specific polypeptide-bound glutamine residues, a reaction that is inhibited by GTP. There is also preliminary evidence that, in situ, calpain and GTP may regulate tTG indirectly by modulating its turnover by the calcium-activated protease calpain. In the present study, the in vitro and in situ proteolysis of tTG by calpain, and modulation of this process by GTP, was examined. tTG is an excellent substrate for calpain and is rapidly degraded. Previously it has been demonstrated that GTP binding protects tTG from degradation by trypsin. In a similar manner, guanosine-5′-*O*-(3-thiotriphosphate) protects tTG against proteolysis by calpain. Treatment of SH-SY5Y cells with 1 n*M* maitotoxin, which increases intracellular calcium levels, resulted in a significant increase in in situ TG activity, with only a slight decrease in tTG protein levels. In contrast, when GTP levels were depleted by pretreating the cells with tiazofurin, maitotoxin treatment resulted in an ~50% decrease in tTG protein levels, and a significant decrease in TG activity, compared with maitotoxin treatment alone. Addition of calpain inhibitors inhibited the degradation of tTG in response to the combined treatment of maitotoxin and tiazofurin and resulted in a significant increase in in situ TG activity. These studies indicate that tTG is an endogenous substrate of calpain and that GTP selectively inhibits the degradation of tTG by calpain. **Key Words:** Tissue transglutaminase—Calpain—Guanosine-5′-*O*-(3-thiotriphosphate)—Transamidation—Maitotoxin—Tiazofurin.
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In its classic role, tissue transglutaminase (tTG) is a transamidating enzyme that catalyzes a calcium-dependent acyl transfer reaction between the γ-carboxamide of a peptide-bound glutamine residue and the ε-amino group of a peptide-bound lysine, or the primary amino group of a polyamine, yielding either an isopeptide bond or a (γ-glutamyl)polyamine bond, respectively (Greenberg et al., 1991). The transamidating activity of tTG is inhibited by GTP, an effect that is reversed by an intrinsic GTPase activity of tTG (Achyuthan and Greenberg, 1987; Lee et al., 1989). In addition to its transamidating activity, tTG also appears to function as a signal transducing GTP-binding protein (Nakaoka et al., 1994) and couples activated α_{1B} adrenoreceptors to phospholipase Cδ, resulting in stimulation of this effector enzyme (Nakaoka et al., 1994; Feng et al., 1996).

The expression of tTG is highly regulated and various factors have been shown to induce expression of tTG in different tissue types and cell lines, including cyclic AMP (Perry et al., 1995), interleukin-6 (Suto et al., 1993), and NFκB activation (Mirza et al., 1997). However, in most cell types, retinoids appear to be the most effective inducers of tTG expression (Davies et al., 1985; Piacentini et al., 1992; Kosa et al., 1995; Benedetti et al., 1996; Nagy et al., 1996). TG activity has been shown to increase approximately two- to threefold during the maturation of mouse brain (Maccioni and Seeds, 1986), and it has been well documented that tTG levels increase significantly during apoptosis in numerous cell types (el Alaoui et al., 1992; Piacentini et al., 1996; Nemes et al., 1997).

The in vitro regulation of the transamidating activity by calcium and GTP has been thoroughly examined and well established. Calcium is absolutely required for activity (Hand et al., 1985; Lai et al., 1997), and in vitro GTP noncompetitively inhibits the transamidating activity of tTG and protects tTG against trypsin digestion (Achyuthan and Greenberg, 1987). It also has been demonstrated that tTG has GTPase activity, with the GTPase active site located in the N-terminal, distal and independent from the transamidating activity site (Lee et al., 1993; Lai et al., 1996).

Although the in vitro regulation of transamidation

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Abbreviations used: CBZ-LLY-DMK, *N*-carbobenzyloxy-leucyl-L-leucyl-L-tyrosine diazomethyl ketone; CIP, calpain inhibitor peptide I (*N*-acetyl-Leu-Leu-norleucinal); GTP-γ-S, guanosine-5′-*O*-(3-thiotriphosphate); MTX, maitotoxin; RA, retinoic acid; TG, transglutaminase; tTG, tissue transglutaminase.

has been thoroughly examined, few studies have focused on the endogenous regulation of tTG. One study measured TG activity in permeablized cells in response to exogenously added calcium and nucleotides, but endogenous regulating mechanisms could not be studied in this system (Smethurst and Griffin, 1996). Recently, our laboratory presented evidence that tTG is likely a tightly controlled enzyme and that it is regulated by both calcium and GTP in situ (Zhang et al., 1998). During the course of these studies, preliminary evidence was obtained indicating that tTG activity may be regulated indirectly, as well as directly, by calcium and GTP through the calcium-dependent thiol-protease calpain.

Calpains are a family of proteases that require both calcium and a reduced environment for activity. The two homologous isoforms, μ-calpain and m-calpain, have been classically distinguished on differences in their in vitro calcium requirements for half-maximal activity with μ-calpain requiring significantly less calcium than m-calpain (Mellgren, 1987). The homologous isoforms of calpains have been shown to proteolyze several protein substrates, often generating large, stable breakdown products. Certain transcription factors (Pariat et al., 1997; Steff et al., 1997), cyclin D1 (Choi et al., 1997), and several cytoskeletal proteins (Suzuki, 1987; Johnson et al., 1989; Goll et al., 1992) have all been shown to be calpain substrates. Calpains are heterodimers consisting of an 80-kDa catalytic subunit and a 30-kDa regulatory subunit. Although intact μ-calpain has been shown to be active in vitro (Guttmann et al., 1997), autolysis lowers the calcium concentration required for half-maximal activity (Goll et al., 1992). The autolytic event involves the "self-removal" of the N-terminal of both the 80-kDa subunit, resulting in a 78-kDa and subsequently a 76-kDa form, and conversion of the 30-kDa subunit to ~18 kDa (Dayton, 1982; Inomata et al., 1988). In healthy cells, calpain is active; but the autolysed forms are not observed, and therefore it has been hypothesized that calpain autolysis may occur predominantly in pathological conditions (Johnson and Guttmann, 1997). μ-Calpain was the focus of this study, as it is prevalent in neurons and activated by concentrations of calcium that have been shown to occur in the cell (Llinas et al., 1992; Petrozzio et al., 1995; Guttmann et al., 1997).

In the present study, the interrelationship between tTG and calpain activity was examined. Preliminary data indicated that tTG may be an endogenous substrate of calpain, and that calpain may be involved in the regulation of tTG transamidating activity in situ by modulating intracellular tTG levels (Zhang et al., 1998). Here we provide more extensive experimental evidence, both in situ and in vitro, to support the hypothesis that tTG is a substrate of calpain.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin, retinoic acid (RA), Tween 20, *o*-phenylenediamine dihydrochloride, dimethyl sulfoxide, and guinea pig liver tissue transglutaminase were purchased from Sigma; phenylmethylsulfonyl fluoride, sodium dodecyl sulfate, fluorescein isothyocyanate-conjugated streptavidin, and dithiothreitol were purchased from Boehringer Mannheim; and the enhanced chemiluminescence (ECL) reagents were purchased from Amersham. 5-(Biotinamido)pentylamine, horseradish peroxidase-conjugated streptavidin and BCA protein assay reagents were purchased from Pierce, and *N*-carbobenzyloxy-L-leucyl-L-leucyl-L-tyrosine diazomethyl ketone (CBZ-LLY-DMK) was from Molecular Probes. *N*-Acetyl-Leu-Leu-norleucinal (calpain inhibitor peptide I, CIP), guanosine-5′-*O*-3-thiotriphosphate (GTP-γ-S), μ-calpain, and Nonidet P-40 were purchased from Calbiochem. Maitotoxin (MTX) was from Alexis. The tau monoclonal antibodies Tau-1 and Tau-5 were from Dr. L. Binder, and the anti-μ-calpain monoclonal antibody was from Dr. J. Elce. Tiazofurin was a gift from the National Cancer Institute. RPMI 1640 was purchased from Cellgro; penicillin/streptomycin and horse serum were from GibcoBRL; and fetal clone II was purchased from Hyclone. The tTG monoclonal antibody 4C1 was produced by the Hybridoma Core Facility at the University of Alabama at Birmingham (Johnson et al., 1997); horseradish peroxidase-conjugated goat anti-mouse IgG was purchased from Bio-Rad. Fura-2 was from Tef Labs. The longest form of human recombinant tau (Goedert et al., 1989) was expressed in bacteria and purified as previously described (Fleming et al., 1996).

Cell culture

Human neuroblastoma SH-SY5Y cells were maintained on Corning dishes in RPMI 1640 medium supplemented with 20 m*M* glutamine, 10 U/ml penicillin, 100 μg/ml streptomycin, 5% fetal clone II serum, and 10% horse serum. For differentiation, the percentage of fetal clone serum and horse serum in the media were reduced to 1 and 4%, respectively. To initiate differentiation, cells were placed in the low serum medium containing RA at a final concentration of 20 μM. The differentiating medium supplemented with 20 μM RA was replaced every 48 h until day 6. All in situ experiments were performed on subconfluent cultures that had been treated with RA for 6 days.

In vitro proteolysis assay

To quantitatively examine the factors that modulate the proteolysis of tTG by μ-calpain, in vitro proteolysis assays were performed with purified tTG and μ-calpain.

To examine the relationship between the concentration of μ-calpain and tTG, assays were performed in a total reaction volume of 200 μl containing 200 p*M* tTG, 100 μM $CaCl_2$, 2 m*M* dithiothreitol, 40 m*M* HEPES buffer, pH 7.4, and μ-calpain (80 kDa) at a final concentration from 0 to 200 p*M*. The reactions were initiated by the addition of $CaCl_2$. After incubating at 37°C for 5 min, the reactions were stopped by removing 10-μl aliquots and adding them to 90 μl of 2× sodium dodecyl sulfate stop solution, followed by incubation in a boiling water bath. Samples containing 15 ng of tTG were electrophoresed in each lane on an 8% sodium dodecyl sulfate–polyacrylamide gel and transferred to a nitrocellulose. The resulting blots were probed with the monoclonal tTG antibody 4C1 (0.25 μg/ml), followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG, and visualization by using standard ECL protocols. The quantitative analyses of the immunoblots were performed by using a Bio-Rad GS-670 imaging densitometer, and the data were expressed as percentages of the initial substrate.

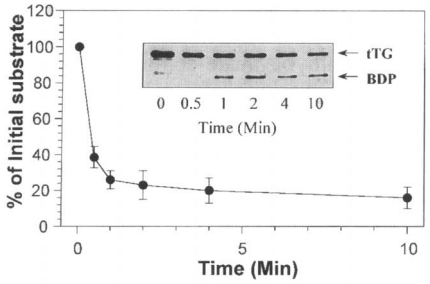

FIG. 1. Time course of tTG proteolysis by μ-calpain. tTG (200 pM) was incubated with 100 pM calpain in the presence of 100 μM $CaCl_2$ for the times indicated. Samples containing 15 ng of tTG were resolved on an 8% sodium dodecyl sulfate–polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with 4C1 (0.25 μg/ml). **Inset:** A representative immunoblot. tTG is rapidly degraded by μ-calpain with the appearance of large breakdown product (BDP). Data are presented as percentages of the tTG remaining at each time point compared with the amount present at time 0. Data are expressed as mean ± SEM values (n = 3 separate experiments).

To determine the effects of calcium concentration on the μ-calpain proteolysis of tTG, the reaction conditions as described above were used, except that the μ-calpain concentration was fixed at 100 pM and calcium concentrations were varied from 0 to 2,000 μM. The reactions were stopped and the samples were analyzed as described above.

To examine time-dependent tTG degradation by calpain, the reaction included 100 pM μ-calpain, 200 pM tTG, and 100 μM $CaCl_2$; other reaction conditions were the same as above. The reactions were stopped at the time points as indicated, and the samples were analyzed as above.

Examination of the inhibition of calpain-mediated proteolysis of tTG was performed by using CBZ-LLY-DMK, CIP, or GTP-γ-S. The reaction contained 40 mM HEPES, pH 7.4, 2 mM dithiothreitol, 100 pM μ-calpain, 200 pM tTG, 100 μM $CaCl_2$, and different concentrations of inhibitor as indicated in Results. Inhibitors were preincubated in the reaction mixture for 1 min at 37°C, and the reactions were initiated by addition of 100 μM $CaCl_2$, and further incubation for an additional 5 min at 37°C. The reactions were stopped and processed as described above. As a control, the calpain-mediated proteolysis of recombinant tau (Litersky et al., 1993) was examined under the same conditions as described for tTG.

In situ tTG activity assay

For in situ tTG activity studies, SH-SY5Y cells that had been treated with RA for 6 days were labeled for 1 h with 2 mM 5-(biotinamido)pentylamine in differentiation medium before treatment with the indicated drugs or vehicles (controls). Tiazofurin was dissolved in water. MTX, CBZ-LLY-DMK, and CIP were dissolved in dimethyl sulfoxide. The maximal dimethyl sulfoxide concentration to which the cells were exposed was <0.5%. Cells were treated with the drugs as indicated, harvested in a homogenizing buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of aprotinin, leupeptin, and pepstatin) and sonicated on ice. In situ transglutaminase (TG) activity was measured, using a microplate assay as previously described (Zhang et al., 1998). The activity of tTG in situ was calculated as a percentage of basal activity (i.e., no drug additions) within a given group of samples. To evaluate the level of tTG in cells after treating with the different reagents, extracts from cells were prepared and quantitatively immunoblotted with 4C1 (Zhang et al., 1998).

Measurement of intracellular calcium levels

Intracellular calcium levels were measured in cultured cells using fura-2 essentially as described previously (Zhang et al., 1998).

All data were analyzed by using Student's t test, and values were considered significantly different when $p < 0.05$.

RESULTS

tTG is a good in vitro substrate of μ-calpain

Previous studies indicated that tTG may be a substrate of calpain (Zhang et al., 1998). Therefore, in initial studies, the in vitro proteolysis of tTG by μ-calpain was examined. Incubation of 200 pM tTG in the presence of 100 pM μ-calpain and 100 μM $CaCl_2$ resulted in the rapid degradation of tTG (Fig. 1). By 0.5 min, >60% of the tTG had been degraded, and after 10 min only ~15% of the substrate remained. Proteolysis of tTG by μ-calpain resulted in the formation of a large stable breakdown product, which is typical of the proteolytic cleavage of substrates by calpain (Fig. 1, inset) (Johnson and Guttmann, 1997). It should be noted that because tTG and calpain are both calcium activated there is the potential for the cross-linking of tTG during the reaction. However, maintaining the concentration of tTG at ≤200 pM prevented any detectable cross-linking from occurring. Further, inactivation of tTG by alkylating the active site cysteine with iodoacetamide (Guttmann et al., 1995) did not alter the kinetics of tTG degradation by calpain (data not shown). Incubation of 200 pM tTG with concentrations of μ-calpain ranging from 0 to 200 pM demonstrated that tTG was maximally degraded by 100 pM μ-calpain (Fig. 2). Increasing the concentration of μ-calpain further did not increase the amount of tTG that was degraded. Calcium concentration-dependent experiments revealed that 5 μM calcium was sufficient to initiate the proteolysis of tTG by μ-calpain

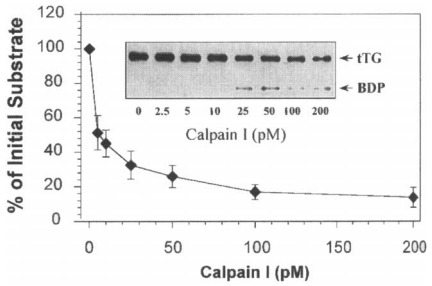

FIG. 2. Calpain concentration-dependent tTG proteolysis. tTG (200 pM) was incubated with 0–200 pM calpain in the presence of 100 μM $CaCl_2$ for 5 min. **Inset:** A representative immunoblot with the breakdown product (BDP) indicated. Data are expressed as mean ± SEM values (n = 3 separate experiments).

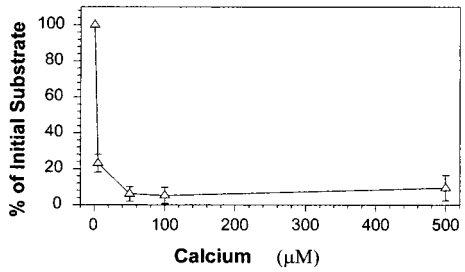

FIG. 3. Calcium concentration-dependent tTG proteolysis. tTG (200 p*M*) was incubated with 100 p*M* calpain in the presence of 0–500 μM $CaCl_2$ for 5 min at 37°C. Data are expressed as mean ± SEM values (n = 3 separate experiments).

(Fig. 3 and Fig. 4A). It is interesting that although tTG was significantly degraded by μ-calpain at calcium concentrations of 5 to 10 μM (Fig. 3 and Fig. 4A), autolytic conversion of μ-calpain was not observed at these calcium concentrations (Fig. 4B). These findings are consistent with previous data (Guttmann et al., 1997; for review, see Goll et al., 1992; Johnson and Guttman, 1997) and indicate that μ-calpain is active in its intact 80-kDa form.

μ-Calpain inhibitors effectively protect the proteolysis of tTG in vitro

CBZ-LLY-DMK and CIP are potent inhibitors of calpain (Bronk and Gores, 1993; Saito and Nixon, 1993; Mellgren et al., 1994) and both inhibited tTG proteolysis by μ-calpain (Fig. 5A and B). Previous studies have demonstrated that, in vitro, GTP binds to tTG and inhibits its proteolysis by trypsin (Achyuthan and Greenberg, 1987). Therefore, the effects of GTP-γ-S on μ-calpain-mediated tTG proteolysis were examined. GTP-γ-S potently inhibited the degradation of tTG by μ-calpain (Fig. 5A) (IC_{50} = 3.5 ± 0.4 μM). In addition, the inhibition of μ-calpain-mediated tTG proteolysis by GTP was similar to that observed with both CBZ-LLY-DMK (IC_{50} = 2.3 ± 0.35 μM) and

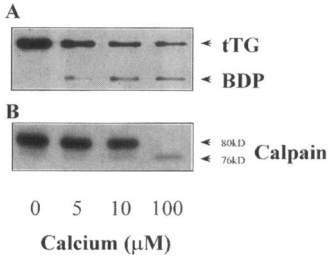

FIG. 4. Effects of calcium concentration on tTG proteolysis and μ-calpain autolysis. tTG (200 p*M*) was incubated with 100 p*M* calpain in the presence of 0, 5, 10, or 100 μM $CaCl_2$ for 5 min at 37°C. Immunoblots were probed with either the anti-tTG antibody 4C1 (**A**) or an anti-μ-calpain antibody (**B**). Significant tTG proteolysis and the formation of breakdown product (BDP) occurred at concentrations of 5 and 10 μM calcium (A), whereas at the same calcium concentrations no calpain autolysis was observed (B).

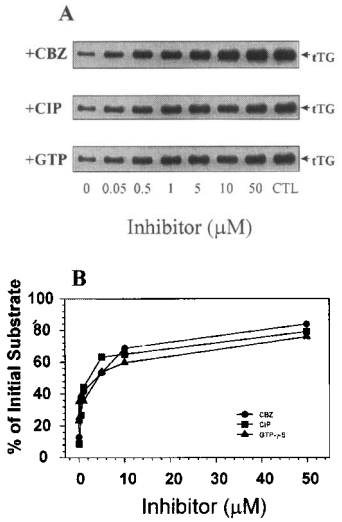

FIG. 5. Inhibition of calpain-mediated proteolysis of tTG by CBZ-LLY-DMK (CBZ), CIP, or GTP-γ-S (GTP). μ-Calpain (100 p*M*) was incubated with 200 p*M* tTG in the presence of 100 μM $CaCl_2$ and the indicated concentrations of CBZ, CIP, or GTP. Samples were also incubated in the absence of inhibitor and the presence of 5 m*M* EGTA as a control (CTL). **A:** Representative immunoblots. **B:** Quantitative analysis from three separate experiments. Data are expressed as percentages of control (CTL) values. The error bars (SEM) are contained within the symbols.

CIP (IC_{50} = 1.7 ± 0.25 μM) (Fig. 5B). To determine that the inhibition of calpain proteolysis of tTG by GTP was selective for tTG, the effects of GTP on calpain-mediated tau proteolysis (Litersky et al., 1993) and calpain autolysis were examined. Although the calpain inhibitor CBZ-LLY-DMK potently inhibited calpain proteolysis of tau (Fig. 6A), GTP-γ-S had no effect on the proteolysis of tau by μ-calpain (Fig. 6B)

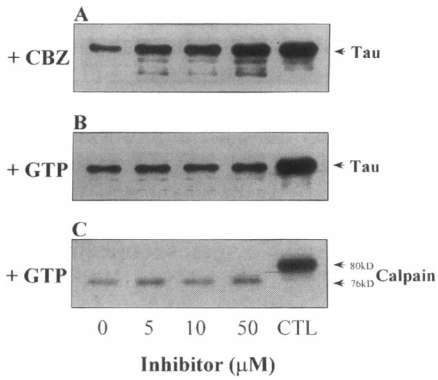

FIG. 6. GTP-γ-S has no effect on the calpain-mediated proteolysis of tau or calpain autolysis. Tau (200 p*M*) was incubated with 100 p*M* μ-calpain in the presence of 100 μM $CaCl_2$ for 30 s at 37°C with the indicated concentration of CBZ-LLY-DMK (CBZ) (**A**) and GTP-γ-S (GTP) (**B** and **C**). Control samples (CTL) contained 5 m*M* EGTA and no calcium, CBZ, or GTP. Immunoblots revealed that CBZ potently inhibited the calpain-mediated proteolysis of tau (A), but GTP-γ-S had no effect on either calpain proteolysis of tau (B) or calpain autolysis (C).

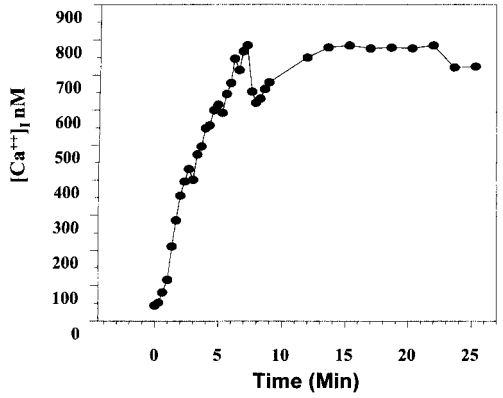

FIG. 7. Calcium response of SH-SY5Y cells to 1 n*M* MTX. MTX was added at time 0 and the $[Ca^{2+}]_i$ was monitored for 25 min. $[Ca^{2+}]_i$ rose rapidly, and after 5 min reached a plateau of 750 n*M* that was maintained for the duration of the experiments.

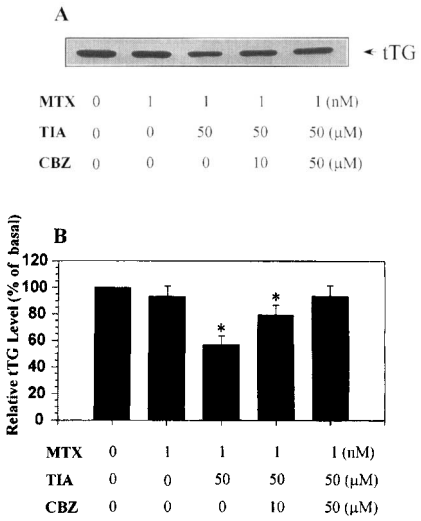

FIG. 8. Inhibition of calpain prevents the decrease in tTG levels in response to depletion of GTP and increased intracellular calcium. SH-SY5Y cells were treated with MTX for 20 min to increase intracellular calcium concentrations, tiazofurin (TIA) for 40 min to decrease GTP levels, or a combination of both (MTX was added during the last 20 min of the incubation with TIA) in the absence or presence of the calpain inhibitor CBZ-LLY-DMK (CBZ), which was added before the addition of MTX or TIA. **A:** Representative immunoblots. **B:** Quantitative data from at least four separate experiments. Data are mean ± SEM values. *$p < 0.05$, compared with basal condition.

or the autolytic conversion of calpain (Fig. 6C). These data suggest that GTP inhibits the degradation of tTG by μ-calpain by specifically interacting with the substrate, tTG.

In situ tTG proteolysis and activity

To examine the proteolysis of tTG in situ, SH-SY5Y cells that had been treated with RA to induce tTG expression were treated with 1 n*M* MTX to activate calpain. MTX has been shown to activate both voltage-sensitive and ligand-gated calcium channels, and potently stimulate calpain activity in SH-SY5Y cells (Wang et al., 1996). Treatments of these cells with 1 n*M* MTX resulted in a rapid rise in intracellular calcium concentrations that was maintained for the duration of the experiment (Fig. 7). In addition, no loss of cell viability, as determined by release of lactate dehydrogenase, was observed with any of the treatment protocols (data not shown) (Zhang et al., 1998).

Incubation of SH-SY5Y cells with 1 n*M* MTX for 20 min resulted in a slight decrease in tTG levels compared with nonstimulated cells (Fig. 8) and, as expected, a significant increase in situ TG activity (Fig. 9). To determine the effects of GTP on the degradation of tTG and TG activity in situ, cells were pretreated for 20 min with tiazofurin, an IMP dehydrogenase inhibitor that effectively decreases intracellular GTP levels (Lee et al., 1985; Zhang et al., 1998). Incubation of SH-SY5Y cells with 50 μ*M* tiazofurin was found previously to deplete intracellular GTP levels by 75–80%, resulting in an intracellular GTP concentration of ~20–40 μ*M* (Smethurst and Griffin, 1996; Zhang et al., 1998). Depletion of GTP by treatment with tiazofurin resulted in a significant decrease in tTG levels in response to MTX (Fig. 8). Concurrent with this decrease in tTG levels in response to MTX and tiazofurin treatment, there was a significant decrease in the in situ TG activity compared with MTX treatment alone (Fig. 9). Addition of the membrane-permeable calpain inhibitor CBZ-LLY-DMK significantly inhibited the degradation of tTG that occurred in response to MTX and tiazofurin treatment (Fig. 8), with an accompanying increase in in situ TG activity (Fig. 9). Identical results were obtained with CIP, another calpain inhibitor (data not shown). These results indicate that tTG is an in situ substrate of calpain. In addition, in the same experimental paradigm, treatment

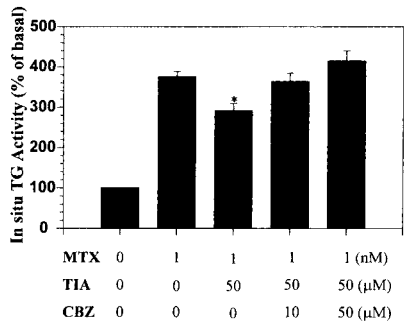

FIG. 9. Depletion of GTP attenuates the MTX-induced increase in in situ TG activity, an effect that is ameliorated by the addition of the calpain inhibitor CBZ-LLY-DMK (CBZ). SH-SY5Y cells were treated with MTX, tiazofurin (TIA), or CBZ as indicated, and in situ TG activity was measured. The activity of tTG in situ is expressed as a percentage of basal activity within a given group of samples. Data are mean ± SEM values and are calculated from at least four separate experiments. *$p < 0.05$, compared with MTX treatment alone.

with MTX also resulted in a significant decrease in tau levels. This effect was unaltered by addition of tiazofurin, but the MTX-stimulated decrease in tau protein was significantly inhibited by the addition of 25 μM CBZ-LLY-DMK (data not shown).

DISCUSSION

The transamidating activity of tTG is likely to play an important role in several different processes including neuronal growth and development (Perry et al., 1995), apoptosis (Nemes et al., 1997), and wound healing (Raghunath et al., 1996). Therefore, it is of crucial importance to understand the mechanisms that regulate the expression and activity of tTG. Previous studies have identified several factors that induce tTG expression in various cell types (Suto et al., 1993; Perry et al., 1995; Mirza et al., 1997), with RA being the best characterized. Nagy et al. (1996) have demonstrated that expression of mouse tTG is mediated by a retinoid response element within the promoter region. It also has been well documented that, in vitro, both calcium and GTP are important regulators of tTG activity. Calcium is absolutely required for activity (Hand et al., 1985; Lai et al., 1997), and in vitro, GTP noncompetitively inhibits the activity of tTG (Achyuthan and Greenberg, 1987). However, the in situ modulation of the transamidating activity by calcium and GTP has not been thoroughly examined. Recently, we demonstrated that, in situ, GTP and calcium play significant roles in directly modulating tTG activity (Zhang et al., 1998). During the course of these studies, evidence was obtained indicating that GTP and calcium may also indirectly modulate the activity of tTG by regulating its turnover by the calcium-activated protease calpain. Therefore, the goal of this study was to extend our initial finding on the involvement of GTP, calcium, and calpain in regulating the in situ activity of tTG.

μ-Calpain proteolyzes numerous substrates including tau (Johnson et al., 1989), cyclin D1 (Choi et al., 1997), certain transcription factors, and several enzymes (for reviews, see Goll et al., 1992; Johnson and Guttmann, 1997). A characteristic of calpain is that it often cleaves substrates in a limited manner, producing large breakdown products. This is most likely because calpain appears to recognize substrates based on various sequence and structural characteristics rather than primary sequence alone (Harris et al., 1989; Melloni and Pontremoli, 1989). It is interesting that calpain is likely to be involved in the activation of another member of the TG family. Epidermal TG exists as a zymogen that is activated on site-specific cleavage (Aeschlimann and Paulsson, 1994). Although several proteases are able to catalyze the activation of the zymogen in vitro, it is likely that, in situ, calpain is the protease that cleaves and activates epidermal TG (Ando et al., 1988; Aeschlimann and Paulsson, 1994). In the present investigation, tTG was found to be an excellent in vitro substrate of μ-calpain, generating a large breakdown product. However, in contrast to epidermal TG, cleavage of tTG by calpain resulted in inactivation.

In situ, GTP-γ-S potently inhibited the degradation of tTG by calpain. tTG is a GTP binding protein with intrinsic GTPase activity. GTP has been shown in vitro to noncompetitively inhibit the transamidating activity of tTG (Lee et al., 1993; Lai et al., 1996) and protect tTG from degradation by trypsin (Achyuthan and Greenberg, 1987). In situ, GTP also inhibits the transamidating activity of tTG (Zhang et al., 1998). In this study, the ability of GTP to protect tTG against proteolysis by calpain in situ was examined. Increasing intracellular calcium concentrations by treating the cells with MTX resulted in a large increase in in situ TG activity. However, if intracellular GTP levels were depleted by treatment with the IMP dehydrogenase inhibitor tiazofurin (Weber et al., 1992; Finch et al., 1993; Zhang et al., 1998), the MTX-stimulated in situ TG activity was significantly less than in the cells treated with MTX alone. This tiazofurin-induced decrease in calcium-activated in situ TG activity was accompanied by concomitant decreases in tTG levels. These decreases in tTG levels and activity were prevented by the addition of the selective calpain inhibitor CBZ-LLY-DMK or CIP. Depletion of GTP had no effect on the MTX-stimulated proteolysis of tau protein, indicating that the effect of GTP is specific for tTG. It is interesting that an earlier study demonstrated that the in situ calcium-stimulated cross-linking of lipocortin I by tTG was significantly enhanced when the cells were pretreated with inhibitors of calpain (Ando et al., 1991). The authors suggested that this was due to a "competition" between tTG and calpain for lipocortin I; however, given the fact that the cells were treated with ionophore for 60 min, degradation of tTG by calpain could contribute to the observed enhancement of lipocortin I cross-linking in the presence of calpain inhibitors.

The present studies clearly indicate that in situ tTG activity is regulated by calcium and GTP both directly and indirectly through the action of calpain. These findings suggest that calpain plays a critical role in maintaining the appropriate levels and activity of tTG within the cell. In addition, the data indicate that in conditions of elevated intracellular calcium levels, GTP more effectively protects tTG against proteolysis compared with its ability to inhibit the transamidating function of tTG. Further studies are required to clarify the physiological and pathological relationship between calpain and tTG.

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