Tissue Transglutaminase Protects against Apoptosis by Modifying the Tumor Suppressor Protein p110 Rb*

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Tissue transglutaminase (TGase) is involved in the regulation of several biological events including cellular differentiation and apoptosis. The expression and activation of TGase are up-regulated in response to retinoic acid (RA), leading to the protection of several cell lines against N-(4-hydroxyphenyl)retinamide (HPR)-induced apoptosis. The anti-apoptotic mechanisms of TGase are poorly understood at this time. We examined the interaction of TGase with the retinoblastoma (Rb) protein, a substrate of TGase that is also implicated in cell survival functions. In cells undergoing HPR-induced apoptosis, Rb is degraded. This degradation is blocked when cells are pretreated with RA, an important regulator of TGase. In vitro studies revealed that TGase protects Rb from caspase-induced degradation in a transamidation-dependent manner. Experiments performed with fibroblasts from Rb−/− mice further demonstrated that the presence of Rb was required for TGase to exhibit anti-apoptotic activity in response to RA treatment. Microinjection of Rb−/− cells with a transamidation-defective TGase mutant and HPR afforded no protection from HPR-induced apoptosis. Taken together, these findings suggest that the ability of TGase to modify Rb via transamidation underlies the ability of TGase to provide protection against apoptotic insults and to ensure that cells remain viable during differentiation.

Tissue transglutaminase or type II transglutaminase (TGase)

is an 87-kDa protein that contains two key catalytic activities, the ability to catalyze protein-amine cross-links and a GTP binding and hydrolysis activity. The transamidation reaction of TGase has been well studied and consists of the Ca2+-dependent formation of covalent bonds between the γ-carboxamide groups of peptide-bound glutamine residues and the primary amino groups of a wide variety of proteins (1, 2). Transamidation has been implicated in a number of biological processes such as axonal regeneration, cellular differentiation, and apoptosis (3–12). Many of the early studies of TGase have identified it as being present in cells and tissues undergoing apoptosis (7–10, 13, 14) and implicated the transamidation activity of TGase as a potentiator of programmed cell death (15, 16). However, there is growing evidence that TGase may not directly mediate apoptosis. TGase−/− mice showed no major developmental abnormalities, and the thymocytes from these mice were no less susceptible to apoptosis than TGase+/+ cells (17, 18). Studies examining the role of TGase in retinoic acid (RA)-mediated signaling (12), as well as studies demonstrating that TGase was required for neurite outgrowth (19), suggest that TGase may exhibit protective effects against apoptotic signals. It has been well documented that RA up-regulates both the expression and transamidation activity of TGase (12, 20–22). We have recently shown that the ability of RA to up-regulate TGase expression and activity is required for the ability of RA to protect against apoptosis induced by a synthetic retinoid analog, all-trans-N-(4-hydroxyphenyl)retinamide (HPR) (12). The TGase-mediated inhibition of apoptosis appeared to involve its transamidation activity and its ability to bind GTP (12), a result that may suggest a role for TGase in more than one anti-apoptotic signaling pathway.

To understand how TGase is acting as an anti-apoptotic factor, we set out to investigate substrates of TGase that are potential regulators of cell death. One such protein was the retinoblastoma gene product (Rb), a well established regulator of the G1/S checkpoint in the cell cycle (23–25). In addition to its role in cell cycle regulation and cellular differentiation, Rb has also been implicated in apoptosis (26). Rb−/− mice are embryonic lethal with significant apoptosis occurring in the developing nervous system and lens of the eye (27–30). The tumor suppressor gene p53 activates a pathway in response to various cellular insults that results in the degradation of Rb (31), a critical event that shifts cells toward apoptosis. The Rb protein was initially identified as a substrate for TGase-mediated transamidation in U937 cells in early stages of apoptosis (11). This finding, when combined with other results showing increased expression of TGase in apoptotic cells (7–10, 13, 14), led to the suggestion that TGase was a pro-apoptotic protein. However, we have found that TGase acts in a protective fashion (12) by modifying proteins during times of cellular stress, thereby creating a window for survival against apoptotic stimuli and enabling cells to continue to grow or differentiate. We show here that the transamidation activity of TGase protects Rb against caspase-catalyzed degradation and that this protective effect is crucial for the anti-apoptotic actions of TGase.

EXPERIMENTAL PROCEDURES

Cell Culture—HL60 cells were maintained in RPMI 1640 medium (Cellgro) with 10% heat-inactivated fetal calf serum (Atlanta Biologicals), 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 g/liter sodium bicarbonate, and 10 mM HEPES in a 37 °C incubator with 5% CO2. Mouse embryonic lung fibroblasts were grown in low glucose Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine

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1 The abbreviations used are: TGase, tissue transglutaminase; G-protein, GTP-binding protein; RA, retinoic acid; HPR, all-trans-N-(4-hydroxyphenyl)retinamide; Rb, retinoblastoma gene product; ELF, embryonic lung fibroblasts; MDC, monodansyl cadaverine; TRIST, Tris-buffered saline plus Tween 20; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; GST, glutathione S-transferase; GFP, green fluorescent protein.

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serum (Atlanta Biologicals) and 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 g/liter sodium bicarbonate, and 10 mM HEPES in a 37 °C incubator with 5% CO₂. Upon stimulation with 5 μM RA (Sigma) or 5 μM HPR (Sigma), the cells were switched to serum-free medium.

Western Blot Analysis—HL60 cells (2 × 10⁶) were incubated in 500 μl of lysis buffer (50 mM Tris- HCl, pH 7.5, 1% Triton X-100, 1 mM EDTA, 1 mM dithiothreitol, 100 μM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml aprotinin, and 150 mM NaCl). Equal amounts of lysate were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes blocked in 5% nonfat dry milk in Tris-buffered saline (20 mM Tris, pH 7.4, 157 mM NaCl, 0.1% Tween 20 (TBST)) for 2 h at room temperature. The membranes were incubated with the primary antibodies (either anti-mouse IgB from Santa Cruz Biotechnology or TGase II Ab-3 mixture (1:1000) (NeoMarkers) overnight at 4 °C. The membranes were washed (three times) with TBST for 5-min intervals and incubated with goat anti-mouse IgG horseradish peroxidase (1:5000) (Amersham Biosciences) for 1 h at room temperature. Membranes were again washed with TBST (three times) for 5 min each, and the proteins bound to the membranes were visualized by enhanced chemiluminescence (Amersham Biosciences).

Purification of Recombinant Proteins—Baculovirus-expressed Rb was purified using E7-peptide (TDLYCEYQLN)-coated Sepharose. Briefly, Sf21 cells infected with Rb were pelleted and resuspended in 1.5 mM HEPES buffer (250 mM NaCl, 5 mM Na₂CO₃, 80 μM pyridoxal-5’-phosphate, 5 μg/ml leupeptin, and 10 μg/ml apro tin) and sonicated four times for 15 s at half-maximal power on ice. The lysate was centrifuged at 4 °C, and the supernatant was transferred to a 1.5-ml Eppendorf tube containing 65 μl of E7-peptide-coated Sepharose in HMG buffer. The mixture was rotated for 30 min at 4 °C and pelleted in a microcentrifuge at 4 °C, and the beads were washed (three times) with 500 μl of HMG buffer. Elution buffer (100 μl) (50 mM Na₂CO₃, 150 mM NaCl, 1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride) was added to the beads and incubated on ice for 30 min. Samples were centrifuged for 30 s at 4 °C, and the supernatant was placed in a new Eppendorf tube and supplemented with 4 μl of HEPES, pH 7.4, and 24 μl of 50% glycerol. The elution process was repeated, and the eluate was pooled and stored at −80 °C. GST-TGase was purified as described previously (32).

In Vitro Caspase Cleavage Assay—TGase-mediated protection of Rb was assayed by incubating insect cell-expressed Rb (10 μg/ml) with GST-TGase (10.5 μg) in 25 μl of transamidation reaction buffer (25 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM KCl, 0.3 mM Na₂HPO₄, 1 mM NaHCO₃, 5 mM glucose, 20 mM dithiothreitol, 0.8% glycerol, 1 mM CaCl₂, and 0.1 mM putrescine). For samples serving as controls, CaCl₂ was omitted. Reactions were incubated at 25 °C for 30 min. Upon completion of the transamidation reaction, the samples were supplemented with 25 μl of caspase cleavage buffer (100 ng of caspase-7, 4 μM EDTA, 0.2% CHAPS, and 20% sucrose) for a total reaction volume of 50 μl. The samples were incubated at 37 °C for 1, 5, 15, and 60 min. Reactions were stopped by the addition of 2× loading buffer, followed by boiling for 5 min. Reaction mixtures were resolved on 8% polyacrylamide gels and analyzed by Western blot as described above.

Microinjection—ELF-7 Rb−/− cells were plated on grid glass coverslips (Bellco) and incubated in serum-free Dulbecco’s modified Eagle’s medium for 18 h. The nuclei of the cells were directly injected with pEGFP (30 ng/μl) alone or in combination with pCMV Rb (30 ng/μl), pTRE HA-TG (30 ng/μl), pTRE HA-TG(C277V) (30 ng/μl), or pCR3.1 V5-TGAC1 (30 ng/μl). Three hundred cells were injected for each treatment.

Apoptotic Assays—5 × 10⁶ ELF Rb−/− and Rb−/− cells were plated onto flame-sterilized coverslips. The next day, cells were incubated in serum-free medium, and apoptosis was induced by the addition of 5 μM HPR for 18 h. Post-treatment, the cells were washed twice with phosphate-buffered saline (2.7 mM KCl, 1.5 mM KH₂PO₄, 137 mM NaCl, and 8.1 mM Na₂HPO₄) and stained with 2.5 μg/ml Hoechst 33258. At least 500 nuclei were counted for each treatment, and the percentage of apoptotic nuclei (condensed and blebbing) was calculated. Apoptosis in cells microinjected with Rb and TGase constructs was determined by counting the number of GFP-expressing cells prior to the addition of HPR. For injected successfully. Ten hours after treatment with HPR, the surviving cells were expressing GFP, and the percentage of cell death was determined. Cell death caused by microinjection alone was controlled by calculating the cell death incurred by cells injected with GFP alone and left untreated for 10 h. This percentage was subtracted from the percentage of cell death induced by a particular condition. Each experimental condition was repeated independently at least three times.
glutamine residues by TGase could block caspase accessibility to the cleavage site on Rb and inhibit caspase cleavage. To test whether TGase is directly involved in preventing the caspase-induced degradation of Rb, as well as determine if the transamination reaction of TGase is required, we looked at the ability of recombinant TGase to protect recombinant Rb from caspase-7 cleavage using a well defined assay system. Rb was incubated with or without TGase in the presence of putrescine, a polyamine that can serve as an amine acceptor for the transamination reaction. The reactions were carried out in the presence or absence of CaCl2, an essential cofactor for the transamination reaction catalyzed by TGase (1, 2). After incubation of TGase with Rb, activated caspase-7 was added for 1, 5, 15, and 60 min, and the reactions were resolved by SDS-PAGE and blotted with an anti-Rb antibody specific for the amino-terminal portion of Rb (allowing the detection of both the full-length and caspase-cleaved forms of Rb). In reactions containing only Rb and TGase, we did not see the appearance of any cleavage products (Fig. 2, lanes 12 and 13). In mixtures containing Rb, TGase, and caspase-7, but lacking calcium, the cleaved form of Rb appeared within 1 min of exposure to caspase-7 (Fig. 2, lane 3). After 60 min of incubation with caspase-7 in the absence of calcium, Rb was completely degraded (Fig. 2, lane 10). However, when the reactions were performed in the presence of calcium, the ability of caspase-7 to degrade Rb was greatly reduced. Even after 1 h of exposure to the caspase, the majority of the Rb that was incubated in the presence of TGase and calcium was still in its native form (Fig. 2, lane 11). When caspase-7 was incubated with Rb in the absence of TGase for 5 min, significant degradation of Rb was observed (Fig. 2, lane 7). Because the TGase-mediated protection of Rb was CaCl2-dependent, we can infer that the TGase-catalyzed transamination activity underlies this protection rather than some type of complex formation between TGase and Rb. These results strongly support the idea that the ability of TGase to modify Rb via transamination is crucial for the protection of Rb from caspase cleavage and may play a major role in the ability of TGase to protect cells against apoptosis.

To determine if the interaction between TGase and Rb was indeed important for TGase-mediated survival, we tested the ability of TGase to protect against HPR-induced apoptosis in cells lacking Rb. ELF cells from either Rb+/− or wild-type (Rb+/-) mice were treated with or without RA from 0 to 3 days. As in other cell types, RA stimulated an increase in TGase expression in both the wild-type and the Rb−/− cells (Fig. 3A). If the ability of TGase to protect cells from apoptosis was linked to its interaction with Rb, one might expect that only the wild-type Rb+/− cells would be resistant to HPR-induced apoptosis in the presence of RA. To investigate the ability of TGase to protect these cells from apoptosis, both wild-type and Rb−/− cells were pretreated for 48 h with 5 μM RA and then treated with or without 5 μM HPR in the presence or absence of the competitive TGase inhibitor MDC. When the wild-type fibroblasts were pretreated with RA and then exposed to HPR, only 21.4% of the cells underwent apoptosis, which is a significantly lower (p = 0.0061) amount of cell death compared with the percentage of cells (41.6%) undergoing apoptosis when wild-type cells were treated with HPR alone (Fig. 3B). Upon adding the competitive inhibitor MDC, 40.9% of the wild-type cells underwent apoptosis, an extent similar to that for cells treated with HPR alone (Fig. 3B), thereby demonstrating that by inhibiting the transamination activity of TGase there is a loss of protection by RA. In the Rb−/− cells, pretreatment with RA did not protect the cells from apoptosis as 38.3% of the cells were dead after 12 h of exposure to HPR (Fig. 3B), a value not statistically different (p = 0.199) from the 48.7% of Rb−/− cells that underwent apoptosis after a 12-h exposure to HPR alone. These results suggest that the anti-apoptotic actions of TGase may rely on its ability to transamidate Rb.

To demonstrate that the combination of TGase activity and Rb is required for the protective effects by TGase, Rb−/− fibroblasts were microinjected with DNA encoding either the wild-type TGase or a catalytically inactive form of TGase (TG(C277V)) or a truncated version of TGase that lacked the most carboxyl-terminal β-barrel (TGAC1), in combination with DNA encoding Rb and GFP (as a marker for injection). Eighteen hours after injection, the cells were treated with or without 5 μM HPR for 8 h, and the percentage of injected cells undergoing apoptosis was determined. Cells that either were not injected or were injected with GFP alone, showed high levels of apoptosis when treated with HPR (63.6% of the un.injected cells were apoptotic, and 55.5% of the cells injected with GFP were apoptotic (Fig. 4)). Cells injected with RA alone also showed relatively high levels of apoptosis when treated with HPR (Fig. 4). Interestingly, cells injected with Rb and wild-type TGase, when treated with HPR, showed a significantly lower level of apoptosis with only 28.8% of the cells undergoing programmed cell death. Cells injected with the transamination-defective TGase mutant, TG(C277V), and Rb were afforded no protection and exhibited high levels of apoptosis (47.8%), comparable with

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**Fig. 2.** TGase protects Rb from caspase-7 cleavage in a Ca2+-dependent manner. Recombinant Rb (10.5 μM) was incubated with GST-TGase (10.5 μM) and caspase-7 (100 ng) in the presence (lanes 2, 4, 6, 9, and 11) or absence (lanes 1, 3, 5, 8, and 10) of 1 mM CaCl2 from 0 to 60 min. The reactions were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with antibodies to the amino terminus of Rb to detect the presence of the intact and caspase-cleaved forms of Rb.

**Fig. 3.** RA protects Rb+/− fibroblasts from HPR-induced apoptosis but not Rb−/− fibroblasts. A, RA-mediated expression of TGase in wild-type (Rb+/−) and Rb−/− cells. Western blot analysis of wild-type and Rb−/− cells treated with RA. B, HPR-stimulated apoptosis in wild-type and Rb−/− cells. Wild-type and Rb−/− cells were treated with or without 5 μM RA for 2 days in serum-free media and then treated with or without 5 μM HPR and 10 μM MDC for 12 h. Cells were fixed with 3.7% formaldehyde and stained with H33258. Pyknotic nuclei were counted (500 nuclei/treatment) to determine a percentage cell death. Results are presented as the mean ± S.D. of at least three experiments.
In summary, our results demonstrate that an interaction between TGase and Rb, which requires a transamination-competent TGase, is necessary for the ability of TGase to inhibit apoptosis. These findings would argue for the actions of TGase as an anti-apoptotic factor. It is quite possible that the up-regulation of TGase often observed in cells undergoing apoptosis (7–10, 13, 14) represents a cellular regulatory mechanism to block or delay the onset of cell death, rather than reflecting a direct participation by TGase in programmed cell death. Apoptotic signaling pathways are tightly regulated, and there are often several levels of regulation necessary for protection against cell death. By modifying proteins like Rb, TGase may extend the lifetime of the cell, slowing the process of programmed cell death and allowing the appropriate anti-apoptotic pathways to become activated. We are currently working on identifying the sites on Rb that are modified by TGase with the expectation that we will find a pattern or motif common to other targets of TGase involved in anti-apoptotic signaling.

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