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Transglutaminase 2 Kinase Activity Facilitates Protein Kinase A-induced Phosphorylation of Retinoblastoma Protein*[S]

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Transglutaminase 2 (TG2, tissue transglutaminase) is a multifunctional protein involved in cross-linking a variety of proteins, including retinoblastoma protein (Rb). Here we show that Rb is also a substrate for the recently identified serine/threonine kinase activity of TG2 and that TG2 phosphorylates Rb at the critically important Ser[780] residue. Furthermore, phosphorylation of Rb by TG2 destabilizes the Rb·E2F1 complex. TG2 phosphorylation of Rb was abrogated by high Ca^{2+} concentrations, whereas TG2 transamidating activity was inhibited by ATP. TG2 was itself phosphorylated by protein kinase A (PKA). Phosphorylation of TG2 by PKA attenuated its transamidating activity and enhanced its kinase activity. Activation of PKA in mouse embryonic fibroblasts (MEF) with dibutyryl-cAMP enhanced phosphorylation of both TG2 and Rb by a process that was inhibited by the PKA inhibitor H89. Treatment with dibutyryl-cAMP enhanced Rb phosphorylation in MEF$^{tg2+/+}$ cells but not in MEF$^{tg2-/-}$ cells. These data indicate that Rb is a substrate for TG2 kinase activity and suggest that phosphorylation of Rb, which results from activation of PKA in fibroblasts, is indirect and requires TG2 kinase activity.

TG2 is a ubiquitous enzyme that is part of a family of evolutionarily conserved proteins that mediate post-translational protein modifications and protein-protein interactions (1, 2). The best characterized function of TG2 is its calcium-dependent transamidating acyltransferase activity that cross-links glutamine with lysine residues in the same proteins, resulting in polymerization or with lysine residues in other proteins, resulting in protein cross-linking. It has also been reported to be a protein-disulfide isomerase (3), to function as a novel G protein (4), and to have a role in transmitting signals from classical G-coupled receptors such as the α_{1B}-adrenergic receptor (5).

We recently identified TG2[3] as a serine/threonine kinase present in human breast cancer cell membranes that is responsible for the phosphorylation of insulin-like growth factor (IGF)-binding protein-3 (IGFBP-3), (6), an IGF-binding protein that, in addition to its function as an IGF carrier protein, also has IGF-independent pro-apoptotic effects in various cell types (7). The K_m and V_{max} for TG2-induced phosphorylation of the IGFBP-3 were in the physiological range (6) and similar to that described for other kinase (8, 9).

Activation of TG2 gene expression is an early event in the apoptosis process, in response to a wide variety of apoptotic stimuli (10–12), as well as being involved in specific forms of diabetes (13). Evidence now exists that TG2 can both promote apoptosis and protect against cell death depending upon cell type, apoptotic stimulus, and subcellular localization of TG2 (14, 15). TG2 activity catalyzing the formation of $\epsilon.\gamma$ glutamyl-lysine cross-links between polypeptide chains and the formation of apoptotic bodies seems important in the later stages in the apoptotic process (16). The rise in intracellular Ca^{2+} that accompanies many pro-apoptotic stimuli may be responsible for the activation of the cross-linking activity of TG2 (17, 18). Mammalian TG2 can bind and hydrolyze both ATP and GTP (19), and GTP has been reported to inhibit the cross-linking activity of TG2 (20).

In some cell types cytosolic TG2 appears to be pro-apoptotic, whereas nuclear localization of a mutant TG2 devoid of transamidating activity protects against apoptosis (21). The protective effect of TG2 in this experimental paradigm appears to require Rb but does not involve polymerization of Rb (21). In other circumstance cross-linking of Rb appears to be important in the anti-apoptotic effect of TG2 (22). Thus the interaction between TG2 and Rb is complex and remains poorly understood.

Rb is involved in regulating the expression of genes that favor cell cycle progression and suppressing the expression of genes involved in apoptosis, and its phosphorylation plays an important role in the regulation of its function. Because Rb has been shown to be a substrate for the transamidating activity of TG2, we investigated whether TG2 could also phosphorylate Rb.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

† This article is dedicated to the memory of Prof. Liam J. Murphy (1950–2006) who passed away during the preparation of this manuscript.

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[3] The abbreviations used are: TG2, transglutaminase 2; IGF, insulin-like growth factor; IGFBP-3, insulin-like growth factor-binding protein-3; Rb, retinoblastoma protein; PKA, protein kinase A; BrdUrd, bromodeoxyuridine; MEF, mouse embryonic fibroblast; CDK, cyclin-dependent kinase; ERK, extracellular signal-regulated kinase.

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MATERIALS AND METHODS

Antibodies and Proteins—IGFBP-3, goat polyclonal TG2 antibody, and the $Rb^{773-928}$ fragment were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Full-length recombinant Rb protein was purchased from QED Bioscience Inc. (San Diego). Recombinant His-tagged human TG2, from Roboscreen Germany, was used for most experiments unless otherwise stated. Recombinant human TG2 expressed in *Escherichia coli* was purchased from Neomarkers Inc. (Fremont, CA). Rabbit polyclonal anti E2F1, rabbit polyclonal anti-Rb, rabbit polyclonal anti-Rb (Ser^{780}), and rabbit polyclonal PKAα antibodies were obtained from Santa Cruz Biotechnology. PKA catalytic subunit was from New England Biolabs (Ipswich, MA). The PKA inhibitor H89 was from Calbiochem. Dibutyryl-cAMP, guinea pig liver TG2, mouse monoclonal anti-phosphothreonine (clone PTR-8), anti-phosphoserine (clone PSR-45), anti-phosphotyrosine (clone PT-66) antibodies, and all other reagents unless otherwise stated were obtained from Sigma-Aldrich.

In-gel Kinase Assay—Human recombinant TG2 (500 ng) was separated by electrophoresis on 10% SDS-PAGE to which 50 μg/ml IGFBP-3 was added just prior to polymerization. After electrophoresis, SDS was removed from the gel by washing twice for 30 min in 20% 2-propanol in 50 mM Tris-HCl (pH 8.0) at room temperature. The enzyme was denatured by incubating the gel for 20 min at room temperature in 6 M guanidine-HCl in 50 mM Tris-HCl (pH 8.0) containing 5 mM 2-mercaptoethanol. Subsequently, the protein was renatured in six changes in a buffer containing 50 mM Tris-HCl (pH 8.0), 0.04% Tween 20, 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM dithiothreitol at 4 °C for 18 h. The gel was preincubated in assay buffer (40 mM Hepes (pH 7.4), 2 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM EGTA) for 30 min at room temperature and then incubated in assay buffer containing 50 μM ATP and 50 μCi of [γ-^{32}P]ATP for 2 h at 30 °C. After incubation, the gel was washed five times for 5 min each at room temperature in 5% trichloroacetic acid containing 1% sodium pyrophosphate. The gel was stained with Coomassie Blue and processed for autoradiography.

In Vitro Kinase Assay—250 ng of phospholipase A2, $Rb^{773-928}$ fragment, PKA, and adenylyl cyclase were incubated with 25 ng of TG2 in kinase buffer (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM ATP, 60 μCi/ml [γ-^{32}P]ATP) in a final volume of 30 μl for 30 min at 30 °C. In some cases $Rb^{773-928}$ was phosphorylated in the presence of Ca^{2+}. In some cases full-length recombinant Rb protein was used. The reaction was stopped by the addition of SDS-PAGE sample buffer, boiled for 5 min, and analyzed on 11% PAGE. Subsequently, gels were dried and processed for autoradiography. In some cases proteins were transferred to nitrocellulose membranes and processed for Western blot.

Western Blotting—Membranes were blocked in 5% skim milk and incubated with the respective primary antibodies for 1 h at room temperature. For detection of phosphospecific amino acids, membranes were incubated with primary antibodies overnight at 4 °C. After incubation, membranes were washed three times in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween 20 (pH 8.0)) and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After washing membranes were analyzed with ECL.

Disruption of Rb•E2F1 Complexes—MCF-7 cells, obtained from ATCC and cultured as described previously (6), were exposed for 12–15 h to 50 μM proteosome inhibitor, *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal. 20 μl of anti-E2F1 antibody was added to 1 ml of MCF-7 cell lysate and incubated for 1 h at 4 °C. 25 μl of protein A-agarose was added and further incubated on a rotating device overnight at 4 °C. After incubation, the pellet was washed four times in ice-cold phosphate-buffered saline and finally suspended in 40 μl of kinase buffer (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM ATP). 200 ng of $Rb^{773-928}$ fragment or full-length Rb protein was added and kept on ice for 1 h. Subsequently 20 ng of recombinant TG2 was added to the reaction mixture and incubated for 30 min at 30 °C. Subsequently tubes were transferred to ice, and Rb that had dissociated from the immobilized E2F1 was washed away by centrifugation. In some cases ATP or TG2 was omitted from the reaction mixture. The fraction of Rb that remained bound to the immunoprecipitate was visualized by immunoblotting with anti-Rb antibody (1:500).

Enzymatic Assay of TG2 Cross-linking and Kinase Activities—To determine the effect of PKA-induced phosphorylation on the activities of the TG2, 250 ng of human recombinant His-tagged TG2 was immobilized on 25 μl of nickel-agarose (Sigma). Free unbound TG2 was removed by centrifugation and washing, and the immobilized TG2 was suspended in 40 μl of PKA assay buffer (containing 20 ng PKA, 100 μM ATP, and 10 mM $MgCl_2$ in 50 mM Tris-HCl (pH 7.4)) and incubated at 30 °C for 15 min. Subsequently tubes were transferred to ice, and the immobilized TG2 was washed three times in Tris-HCl buffer (pH 7.4) to remove the reaction mixture and resuspended in 40 μl of 50 mM Tris-Cl (pH 7.4). 5 μl of nickel-agarose-immobilized phosphorylated and nonphosphorylated TG2 was used for the TG2-induced kinase assay (as above), and 25 μl of resuspension was used for the TG2-induced transamidation reaction. Controls included reactions where ATP or PKA was omitted from the preincubation and where H89 (100 nM), an inhibitor of PKA, was included. In some experiments immobilized phosphorylated TG2 was subsequently dephosphorylated with alkaline phosphatase (3 unit/reaction) prior to testing for cross-linking activity. TG2 cross-linking activity was determined by the formation of hydroxamate from sodium-benzoylcarbonyl (CBZ)-glutaminylglycine and hydroxylamine using L-glutamic acid γ-monohydroxamate as standard (23). In brief, phosphorylated and nonphosphorylated immobilized TG2 were added in a 0.23-ml reaction mix containing 174 mM Tris, 31 mM benzoylcarbonyl-glutaminylglycine, 87 mM hydroxylamine, 8.7 mM glutathione reduced form, and 4 mM $CaCl_2$ and incubated at 37 °C for 15 min. At the end of the incubation absorbance was measured at 525 nm.

Mouse Embryonic Fibroblast (MEF) Culture and Flow Cytometry—MEF obtained from wild type $TG2^{+/+}$ and $TG2^{-/-}$ mice were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37 °C with 5% CO_2 in a humidified atmosphere (24). For cell cycle analysis, cells were collected by trypsinization, pelleted at 800 $\times$ g for 10 min, and fixed in 70% ethanol. DNA content was evaluated by

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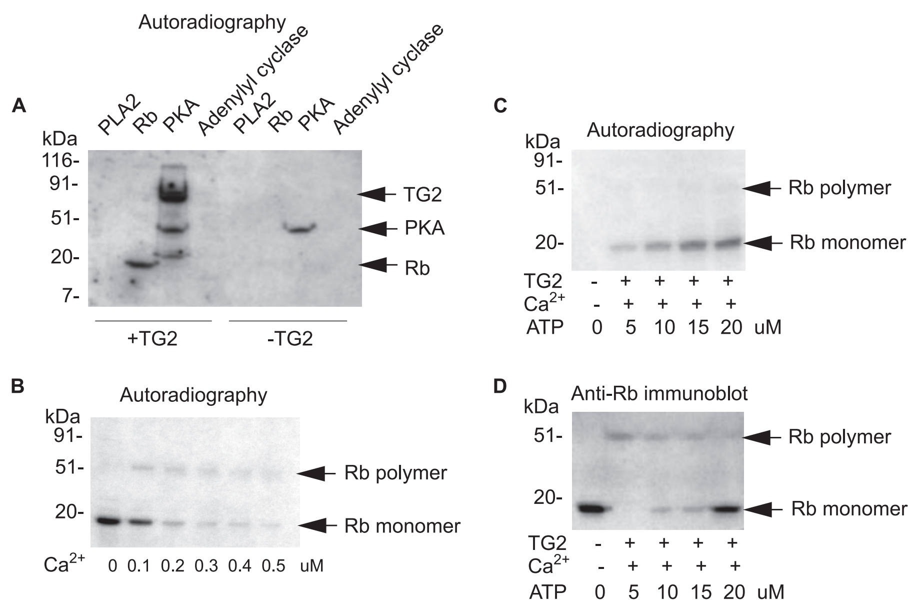

FIGURE 1. **TG2 kinase activity is modulated by Ca^{2+}.** *A*, 250 ng of phospholipase A2 (*PLA2*), $Rb^{773-928}$ fragment, PKA, and adenylyl cyclase were incubated with [γ-^{32}P]ATP (60 μCi/ml) in the presence (25 ng/reaction) and absence of human recombinant TG2 at 30 °C for 30 min and then analyzed by SDS-PAGE and autoradiography. *B*, 250 ng of $Rb^{773-928}$ fragment was incubated with 25 ng of TG2 and [γ-^{32}P]ATP (60 μCi/ml) in the presence of increasing concentrations of Ca^{2+} (0–0.5 μM). After analysis by SDS-PAGE, the radiolabeled Rb fragment was identified by autoradiography. Monomer $Rb^{773-928}$ fragment (~17 kDa) and Rb fragment polymer (~51 kDa) are shown by an *arrow*. *C*, a similar experiment was performed using Ca^{2+} at a concentration of 0.2 μM and increasing concentrations of ATP (0–20 μM). Note that the specific activity of [γ-^{32}P]ATP was maintained constant throughout the experiment. *D*, an anti-Rb (1:500) immunoblot showing the effect of increasing concentrations of ATP on the cross-linking of $Rb^{773-928}$ fragment by TG2 in the presence of 0.2 μM Ca^{2+} concentration is shown. Experiments were repeated for three or more times.

flow cytometry with propidium iodide staining. Twenty thousand events were evaluated by using the CellQuest program (BD Biosciences). Electronic gating (forward scatter area *versus* forward scatter height) was used, when appropriate, to eliminate cell aggregates and to identify the various cell populations. The same method was used to evaluate the cell cycle by using ModFit LT software (Verity Software). $MEF^{tg2+/+}$ and $MEF^{tg2-/-}$ cells were plated at equal concentrations 24 h before pulsing for 30 min with 10 μg/ml bromodeoxyuridine (BrdUrd). Cells were then washed, harvested by trypsinization, and fixed for 30 min at −20 °C in ethanol. DNA was denatured for 30 min using 2 M HCl and 0.5% TX-100 at room temperature and soon after were partially renatured for 10 min in sodium borate buffer (100 mM, pH 8.5). Sample were then incubated for 60 min with anti-BrdUrd antibody (Novocastra Labs, Newcastle upon Tyne, UK) diluted 1:100, washed, and incubated for 30 min with secondary antibody (Alexa fluor 488 goat anti-mouse, Molecular Probes, Eugene, OR) and propidium iodide (100 μg/ml). Cells were measured using a FACScalibur (BD Biosciences) exciting with a 488 nm argon laser. Cell cycle analysis was performed using CellQuest and MODFIT software (BD Biosciences).

Activation of PKA in MEFs—$MEF^{tg2+/+}$ and $MEF^{tg2-/-}$ cells were plated in full media. After 72 h in culture the medium was exchanged for serum-free media, and cells were serum-starved for 6 h. Subsequently cells were treated with dibutyryl-cAMP (100 μM) in the presence and absence of the PKA inhibitor H89 (100 nM). Cell lysates were prepared in 50 mM Tris-HCl buffer (pH 7.5) (120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 mM $NaVO_5$, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). 20 μl of goat polyclonal anti-TG2 (Upstate Biotechnology) or rabbit polyclonal anti-Rb antibody (Santa Cruz Biotechnology) was added to 1 ml of MEF cell lysate and incubated on a rotating device overnight at 4 °C. Protein A-agarose, 25 μl, was added to each tube and the incubation continued for 2 h. At the end of the incubation, the pellet was washed four times in ice-cold phosphate-buffered saline, resuspended in sample buffer, analyzed by SDS-PAGE, and transferred to nitrocellulose membranes. Subsequently membranes were processed for immunoblot with anti-TG2 (1:1000), anti-phosphoserine (1:5000), anti-Rb (1:500) and anti-phospho-Ser^{780} (1:250). After incubation with the respective secondary antibodies-horseradish peroxidase conjugate for 1 h at room temperature, membranes were washed and analyzed with ECL.

RESULTS

TG2 Phosphorylates Rb at Ser^{780}—TG2 kinase activity was originally identified as the kinase responsible for phosphorylation of IGFBP-3 in human breast cancer cell (6). To confirm that the kinase activity was due to TG2 rather than a contaminating, or closely associated, kinase, an in-gel kinase assay was performed using recombinant human TG2 with IGFBP-3 as a substrate (supplemental Fig. 1). A single band was observed, corresponding in size to TG2 identified on the Coomassie-stained gel.

TG2 has been implicated in the cross-linking or activation of a variety of other signaling proteins in apoptosis and cell proliferation. These include Rb (25), ROCK-2 protein kinase (26), mitogen-activated protein kinase (26), adenylyl cyclase and CREB (cAMP-response element-binding protein) (27), phospholipase C (28), and PKA (29). The exact mechanisms whereby TG2 is involved in these signal transduction pathways are unclear. Because protein phosphorylation is central to the activation of many of these signaling pathways, we investigated whether TG2 was able to phosphorylate some of these important signaling proteins (Fig. 1*A*). TG2 was unable to phosphorylate adenylyl cyclase, phospholipase A2, or PKA. The catalytic subunit of PKA undergoes autophosphorylation. Of the proteins examined only $Rb^{773-928}$ fragment was phosphorylated by TG2, which contains 7 of the 16 potential (Ser/Thr) phosphoacceptor sites in Rb (30). This kinase activity was also observed with both guinea pig liver TG2 and human recombinant TG2 expressed in *E. coli* (data not shown). Interestingly we observed

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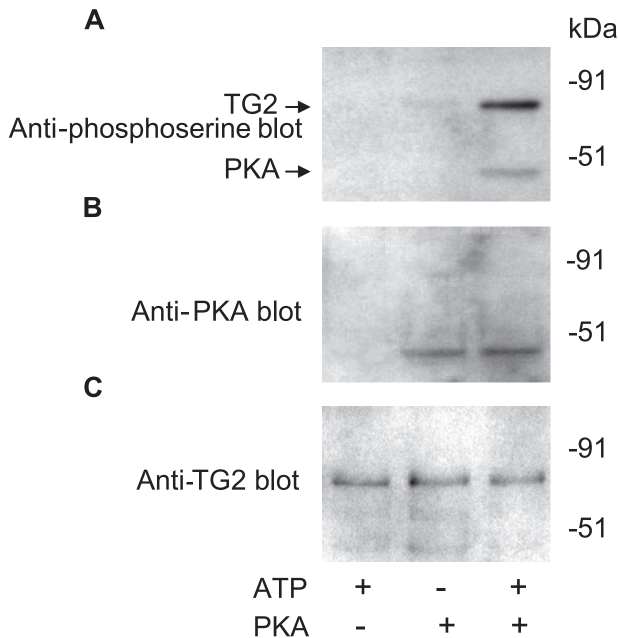

FIGURE 2. **PKA phosphorylates TG2.** Recombinant TG2 (250 ng) was incubated with active PKA (25 ng) and ATP (100 μM) for 30 min at 30 °C. Proteins were separated by 12% SDS-PAGE and processed for Western immunoblot. *A*, anti-phosphoserine (1:5000) immunoblot is shown. TG2 was recognized by anti-phosphoserine only in the presence of ATP and PKA. Active PKA, which undergoes autophosphorylation, was also recognized by the anti-phosphoserine antibody. *B*, the same membrane was reprobed with anti-PKA antibody (1:250). *C*, an anti-TG2 immunoblot (1:1000) is shown with the amount of TG2 equal to that in the control. The experiment was repeated three times.

an ~78-kDa band corresponding to the molecular mass of TG2 in the presence of PKA and ATP in the autoradiograph (Fig. 1*A*). Because there are presumably only two proteins in the reaction, this result suggest that TG2 is phosphorylated by PKA, because this band is not seen in the absence of PKA (Fig. 1*A*).

To further confirm PKA-induced phosphorylation of TG2, TG2 was incubated with PKA and ATP and then processed for immunoblotting with phosphoamino-specific antibodies. Anti-phosphoserine antibody recognizes both PKA and TG2 in the presence of ATP but fail to recognize TG2 in the absence of PKA (Fig. 2*A*), suggesting that PKA-induced phosphorylation of TG2 occurs at the serine residue. Anti-phosphothreonine did not recognize PKA-induced phosphorylated TG2 (data not shown).

The cross-linking activity of TG2 is known to be Ca^{2+}-dependent (12). We confirmed that Ca^{2+} enhanced cross-linking of $Rb^{773-928}$ induced by TG2 (31) but observed that Ca^{2+} inhibited TG2-induced phosphorylation of $Rb^{773-928}$ fragment (Fig. 1*B*). High molecular weight forms of $Rb^{773-928}$ were only lightly phosphorylated compared with monomeric $Rb^{773-928}$. In contrast, ATP appeared to enhance kinase activity and inhibit TG2 cross-linking activity (Fig. 1, *C* and *D*). These data are consistent with the recent report by Lee *et al.* (32) that Ca^{2+} inhibits the GTP binding and GTPase activity of TG2.

Using phosphoamino acid-specific antibodies we demonstrated that TG2-induced phosphorylation occurred predominantly at the serine residues. Phosphotyrosine (data not shown) and phosphothreonine antibodies did not recognize TG2-induced phosphorylated $Rb^{773-928}$ fragment, whereas phosphoserine antibody recognized $Rb^{773-928}$ fragment that had been phosphorylated by TG2 (Fig. 3*A*). Of the 10 potential phospho-

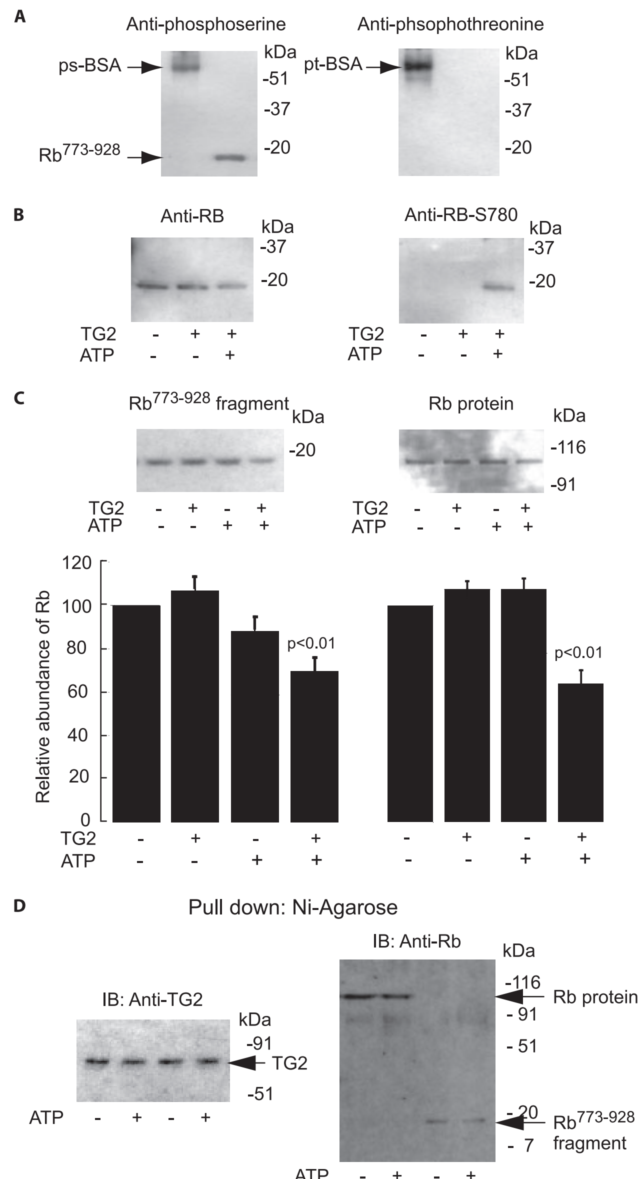

FIGURE 3. **TG2 phosphorylates Ser^{780} in Rb and modulates its interaction with E2F1.** *A*, $Rb^{773-928}$ (250 ng) fragment was incubated with TG2 (25 ng) and ATP (100 μM) at 30 °C for 30 min. Proteins were separated on 12% SDS-PAGE and transferred to a nitrocellulose membrane for immunoblotting. Phosphothreonine antibody failed to recognize phosphorylated $Rb^{773-928}$ fragment, whereas anti-phosphoserine recognized $Rb^{773-928}$ fragment. Phosphoserine-bovine serum albumin (*ps-BSA*) and phosphothreonine-bovine serum albumin (*pt-BSA*) were included as positive controls (*upper panels*). This experiment was repeated three times. *B*, $Rb^{773-928}$ was phosphorylated with TG2 and probed with phospho-specific antibody that recognized Rb phosphorylated at Ser^{780}. The dilutions of the antibodies used were 1:5000, 1:5000, and 1:250 for anti-phosphoserine, anti-phosphothreonine, and Ser^{780}-specific anti-phospho-Rb, respectively. An anti-Rb (1:500) immunoblot is shown as a control. *C*, E2F1 was immunoprecipitated from MCF-7 cell lysates with protein A-agarose and incubated with 200 ng of Rb fragment or with full-length recombinant protein in the presence or absence of TG2 (20 ng) and ATP (100 μM). The reaction mixture was incubated for 30 min at 30 °C and subsequently was transferred to ice. After washing, the beads were boiled in SDS-PAGE loading buffer and the attached proteins resolved by electrophoresis. The residual Rb fragment or full-length Rb protein was quantified by immunoblotting and densitometry. A representative blot is shown (*upper panel*), and the data from four independent experiments are depicted in the *lower panel*. *D*, 200 ng of Rb fragment or full-length Rb protein was incubated with 20 ng of His-TG2 in the presence or absence of ATP (100 μM) for 30 min at 30 °C. Subsequently TG2 was pulled down using nickel-agarose (20 μl). After washing, pulled down proteins were separated by SDS-PAGE and analyzed by immunoblotting (*IB*) using anti-TG2 (1:1000) and anti-Rb (1:500) antibodies. The experiment was repeated three times.

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serine acceptor sites in Rb, five are present in the C-terminal region of the molecule and are present in the $Rb^{773-928}$ fragment. TG2 was able to phosphorylate Ser^{780} (Fig. 3*B*). This region of Rb is involved in binding to the E2F family of transcription factors, which are important in cell cycle progression (31).

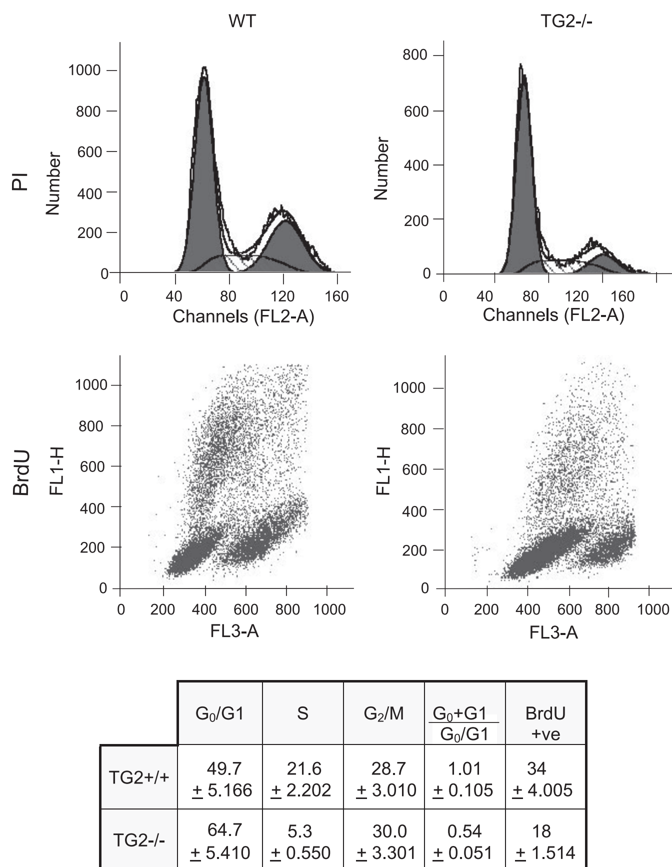
Phosphorylation of Rb by TG2 Disrupts the Rb-E2F1 Interaction—In this experiment, in addition to full-length recombinant Rb protein we have also used $Rb^{773-928}$ fragment because it has been shown that phosphorylation of Rb at the C terminus modulates binding to E2F1. Here we have shown that the C-terminal fragment of Rb is subject to phosphorylation at a critical residue by TG2 kinase activity.

We immunoprecipitated E2F1 from MCF-7 breast cancer cells using anti-E2F1 antibody and protein A-agarose to determine whether TG2-induced phosphorylation of Rb affected the interaction with E2F. The precipitates were incubated with either the $Rb^{773-928}$ fragment or with full-length Rb. Aliquots of the immobilized Rb·E2F1 complex were then incubated with kinase buffer alone, TG2 in kinase buffer, or in TG2 plus ATP. After washing, the amount of residual Rb was quantified by immunoblotting (Fig. 3*C*). TG2 in the presence of ATP significantly reduced the amount of Rb in the pellet by ~33–40%. This reduction in bound Rb was not observed in the absence of ATP. Furthermore, this effect was not due to co-precipitation of another contaminating kinase, because ATP alone had no effect on the amount of Rb in the pellet.

To further confirm that dissociation of Rb from Rb·E2F1 complex was due to TG2-induced phosphorylation of Rb and not to binding to TG2 in the presence of ATP and subsequent loss during washing, His-TG2 was incubated with the Rb fragment and full-length Rb in the presence and absence of ATP. Subsequently TG2 was pulled down using nickel-agarose. Pulled down proteins were analyzed by immunoblotting using anti-TG2 and anti-Rb antibodies. No significant difference in Rb levels was found in the presence or absence of ATP (Fig, 3*D*), suggesting that loss of Rb from Rb·E2F1 complex was due to TG2-induced phosphorylation of Rb and not to increased binding to TG2 in the presence of ATP.

Cell Cycle Progression in $MEF^{tg2+/+}$ and $MEF^{tg2-/-}$ Cells—Because TG2 appeared to modulate Rb-E2F interactions, we evaluate the progression of $MEF^{tg2+/+}$ and $MEF^{tg2-/-}$ cells through the cell cycle phases. A reduced progression through the S phase in $MEF^{tg2-/-}$ cells was demonstrated using flow cytometry and staining with propidium iodide (Fig. 4, *top*). This finding was confirmed by BrdUrd staining (Fig. 4, *middle*).

Phosphorylation of TG2 by PKA Modulates Its Activity—In initial experiments we noted a band corresponding to the molecular weight of TG2 in the presence of PKA and ATP (Fig. 1*A*), and subsequently we showed that PKA-induced phosphorylation of TG2 occurs at the serine residue (Fig. 2). TG2 transamidating activity was measured *in vitro* before and after phosphorylation by PKA (Fig. 5*A*). The incubation of TG2 with either ATP alone or PKA alone had no effect on the transamidating activity. However when both were present together, the transamidating activity was reduced by ~30%. This effect was not seen when H89, an inhibitor of PKA, was included in the incubation (Fig. 5*A*, *lane 5*); alkaline phosphatase dephosphorylation of TG2 phosphorylated by PKA returned the TG2 transamidating activity to normal (Fig. 5*A*, *lane 7*). In contrast phosphorylation of TG2 by PKA enhanced the ability of TG2 to phosphorylate $Rb^{773-928}$ (Fig. 5*B*). Preincubation of TG2 with either ATP or PKA by itself had no effect on kinase activity (Fig. 5*B*, *lanes 2* and *4*), whereas together they significantly increased the ability of TG2 to phosphorylate $Rb^{773-928}$ by $28.6 \pm 1.9\%$ ($p < 0.05$, Fig. 5*B*, *lane 3*). This effect was blocked by H89. We did not test the effects of dephosphorylation of phosphorylated TG2 kinase activity because of the difficulty of fully removing all of the alkaline phosphatase from the assay mix. We used mouse embryonic fibroblasts to investigate the effects of PKA on TG2 activity *in vivo*. Dibutyryl-cAMP was used to activate PKA in MEFs. Dibutyryl-cAMP enhanced the phosphorylation of Rb at Ser^{780} in $MEF^{tg2+/+}$ cells, and this effect was attenuated by the PKA inhibitor (Fig. 6*B*). In $MEF^{tg2-/-}$ cells, no TG2 protein was detected. In $MEF^{tg2+/+}$ cells, under basal conditions some Ser^{780} phosphorylation of Rb was observed. Dibutyryl-cAMP treatment further increased phosphorylation of Rb at Ser^{780}, and this was inhibited by H89. However, in $MEF^{tg2-/-}$ cells under basal conditions, little serine phosphorylation of Rb was observed, and dibutyryl-cAMP did not increase phosphorylation of Rb at Ser^{780} (Fig. 6*B*).



	$G_0/G1$	S	G_2/M	G_0+G1 / G_0/G1	BrdU +ve
TG2+/+	49.7 ± 5.166	21.6 ± 2.202	28.7 ± 3.010	1.01 ± 0.105	34 ± 4.005
TG2-/-	64.7 ± 5.410	5.3 ± 0.550	30.0 ± 3.301	0.54 ± 0.051	18 ± 1.514

FIGURE 4. **A reduced progression through the S phase in $MEF^{tg2-/-}$ cells.** Cell cycle distribution is shown in MEFs obtained from wild type $TG2^{+/+}$ (*left panels*) and $TG2^{-/-}$ (*right panels*) mice. Cells at 70% confluency (identical results were obtained at 50% confluency) show a reduced relative distribution in the S phase (*upper panels* and *table* at *bottom*). This was confirmed also by BrdUrd staining (*lower panels*). The experiment was repeated three times. The *table* shows the mean ± S.E. as a percentage for three independent experiments.

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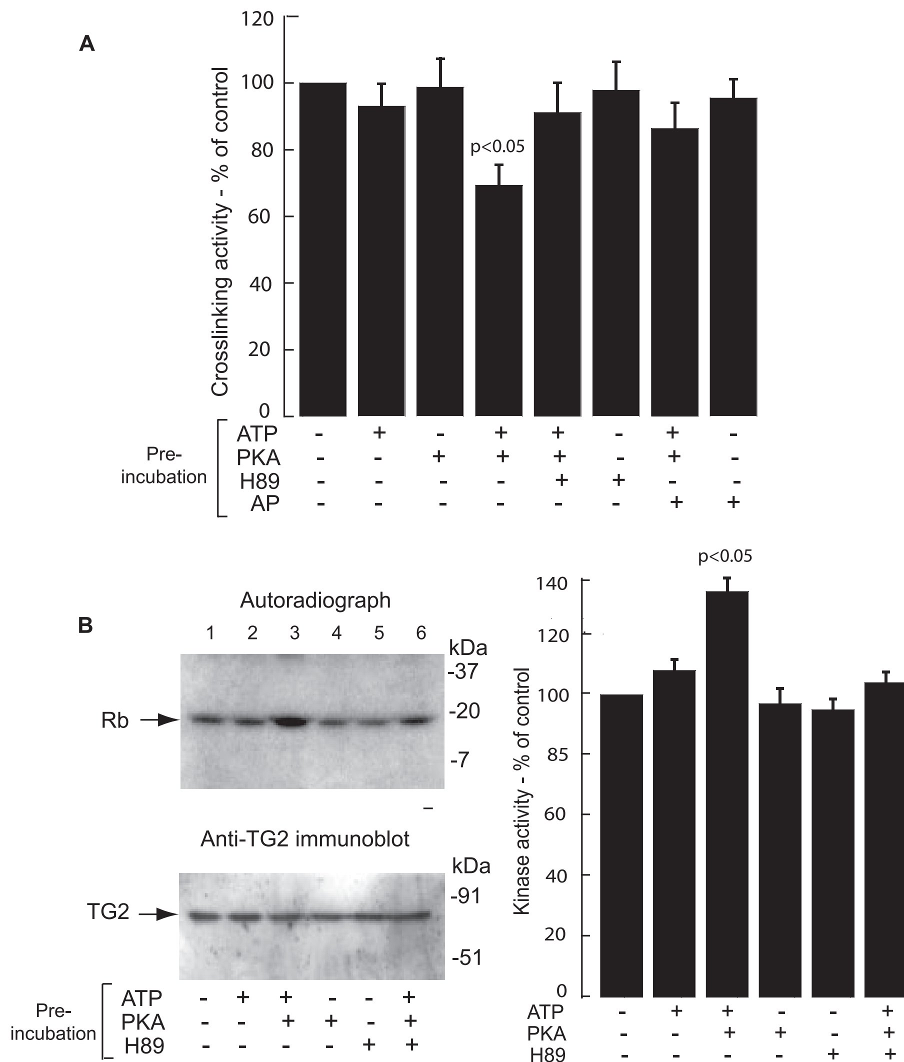

FIGURE 5. **Phosphorylation of TG2 by PKA modulates cross-linking and kinase activities.** *A*, the cross-linking activity of immobilized TG2 was measured as described under "Material and Methods." TG2 was preincubated with PKA in the presence or absence of ATP and H89, an inhibitor of PKA. In some experiments TG2 was subsequently treated with alkaline phosphatase (*AP*). Data represent the mean ± S.E. from three or more experiments. The significant difference between the control and phosphorylated TG2 is indicated. *B*, the ability of TG2 to phosphorylate $Rb^{773-928}$ was measured *in vitro* before and after phosphorylation by PKA. TG2 was phosphorylated by preincubation with PKA in the presence or absence of ATP and H89, as shown. After preincubation the ability of TG2 to phosphorylate $Rb^{773-928}$ protein was assessed using [γ-^{32}P]ATP. Phosphorylated proteins were separated by SDS-PAGE and transferred to membrane for autoradiography. A representative autoradiograph is shown; the data (mean ± S.E., $n = 3$) from three separate experiments were quantified by densitometry. The significant difference between the activity of phosphorylated and nonphosphorylated TG2 is indicated. As a control the same membrane was immunoblotted with anti-TG2 to demonstrate equal amounts of TG2 in the reaction mix.

DISCUSSION

Here we have extended our previous observation that TG2 has intrinsic kinase activity and have demonstrated that this kinase activity is modulated by PKA-induced phosphorylation of TG2. We previously purified an IGFBP-3 kinase activity from human breast cancer cells and identified this kinase as TG2 by tandem mass spectroscopy (6). This activity is apparent in TG2 preparations derived from guinea pig liver and recombinant TG2 expressed in both *E. coli* and insect cells. Furthermore, we have demonstrated in this report that the TG2 kinase activity was detectable using an in-gel kinase assay. These data taken together make it extremely unlikely that kinase activity is due to some contaminating protein in these three preparations. TG2 is known to be able to hydrolyze both GTP and ATP (20). Because it was not possible to demonstrate radiolabeling of IGFBP-3 when [α-^{32}P]ATP was used (6), it would appear that during TG2-induced hydrolysis of ATP the γ-phosphate group is transferred to serine residues in the appropriate substrates. Here we also demonstrated that the transamidating and kinase activities are reciprocally modulated by Ca^{2+}, ATP, and TG2 phosphorylation. We also have provided convincing evidence that PKA can phosphorylate TG2 both *in vitro* and *in vivo*. However, of relevance is an observation of parallel changes in theophylline-induced apoptosis, PKA activation, TG2 expression, and TG2 activation in human epithelial cancer cells (29). Furthermore, an interrelationship between TG2 and PKA-dependent signaling pathways has been demonstrated in differentiation of preosteoblasts in periosteal bone (33). In this setting, TG2 is thought to inhibit PKA-mediated signaling; however, the mechanism involved has not been elucidated (33). A search of reported phosphorylation sites in TG2 using the search engine at www.phosphosite.org revealed that Tyr^{369} has been identified previously as a site of phosphorylation, but the responsible kinase is unknown (34). In human TG1, phosphorylation at Ser^{24}, Ser^{82}, Ser^{85}, and Ser^{92} have been reported, with Ser^{82} being the dominant site of phosphorylation (35). However TG1 and TG2 are quite dissimilar in this region. Recently we have shown that Ser^{216} in TG2 is an important site of phosphorylation by PKA and that phosphorylation at this residue creates a binding site for 14-3-3 protein (36), but the downstream significance of this interaction remains to be elucidated. The surrounding sequence, ^{209}RDCSRRS**S**PVYVGRV^{223}, is conserved in human, bovine, rat, mouse, and guinea pig TG2. 14-3-3 has been shown to interact with various proteins involved in apoptosis, cell cycle, and signaling pathways (37, 38) and to form a complex with retinoblastoma-associated protein RbAp48 (39). The interaction of 14-3-3 with TG2 may be important in the regulation of various functions of TG2, or it may provide a means for cross-talk with other signaling pathways. It therefore warrants further investigation.

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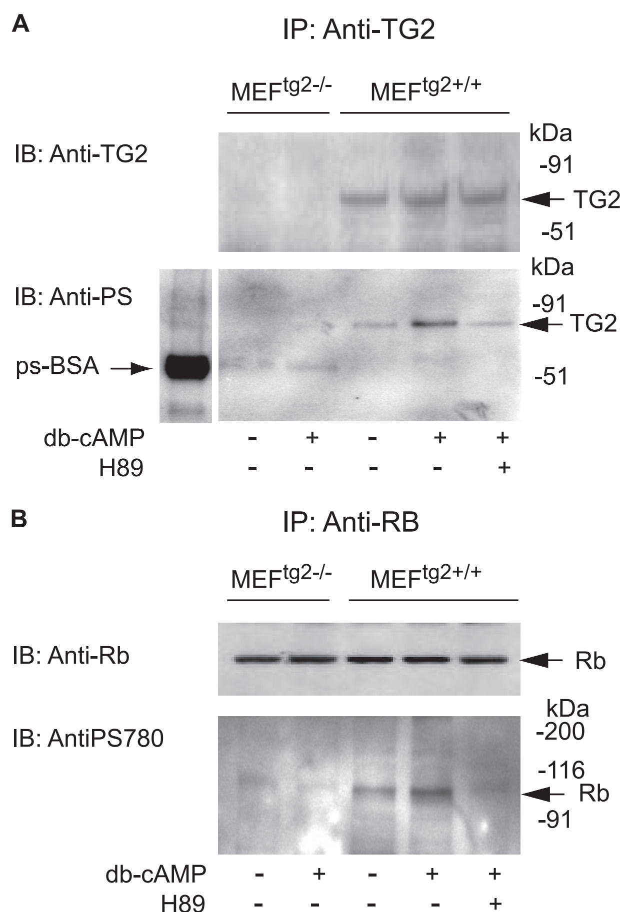

FIGURE 6. **The effect of dibutyryl-cAMP on serine phosphorylation of TG2 and Rb in MEF$^{tg2+/+}$ and MEF$^{tg2-/-}$ cells.** Serum-starved cells were treated with 100 μM dibutyryl-cAMP (*db-cAMP*) and processed for immunoprecipitation as described under "Material and Methods." Subsequently immunoprecipitates (*IP*) were analyzed by immunoblotting (*IB*) with anti-TG2 (1:1000), anti-phosphoserine (*PS*) (1:5000) (in *A*), anti-RB (1:500), or anti-phospho-Ser^{780} (*PS780*) (1:250) (in *B*). Phosphoserine-bovine serum albumin (*ps-BSA*) was included as a positive control (in *A*). Experiments were repeated three times.

TG2 is present in the nucleus as well as in other cellular compartments (40). Nuclear localized TG2 appears to be anti-apoptotic (22). Milakovic *et al.* (21) transfected C277S TG2, a mutant that lacks transamidating activity, and found that this mutant is translocated to the nucleus and has a protective effect against apoptosis. This protective effect correlates with increased interaction with Rb, but under these circumstances the cross-linking of Rb is not observed. It is unclear whether the C277S TG2 mutant retains kinase activity, as this has still to be tested. However, we have shown here that Rb is a substrate for TG2 kinase activity and that TG2 is able to phosphorylate Rb at Ser^{780}, a residue that is important in the binding to the E2F family of transcription factors to Rb. The E2F binding region of Rb spans residues 394 to 864, consisting of the A and B subdomains, an intervening spacer, and a portion of the C terminus (30). The latter portion, sometimes referred to as the C-pocket (41), can bind E2F but also interacts with c-Abl and Mdm2 (42, 43). This E2F binding region contains eight potential phosphoserine acceptor sites, of which five are present in the Rb$^{773-928}$ fragment used here. Mutations of Ser^{807}, Ser^{811}, Thr^{821}, and Thr^{826} do not affect E2F binding (44), suggesting that Ser^{780}, Ser^{788}, or Ser^{795} is functionally important in this regard. Of these residues, Ser^{780} and Ser^{795} appear to be the most important (45, 46). These residues are known to be phosphorylated by a number of kinases including cyclin-dependent kinases (CDK4/6) and ERK1/2 (45, 46). Although the A and B domains of Rb alone appear to be important for E1A/T antigen binding, binding of E2F also requires additional sequences (residues 841–870 in the C terminus (47, 48)); it is phosphorylation in this C terminus that modulates binding of Rb to E2F (31). Furthermore, overexpression of the C-terminal fragment of Rb alone in fibroblasts functions as a dominant negative inhibitor of Rb function (41). Here we have confirmed that E2F1 can indeed bind to the C-terminal fragment, Rb$^{773-928}$, and have also demonstrated that phosphorylation of this fragment or full-length Rb protein by TG2 is associated with destabilization of the Rb·E2F1 complex. It has been reported previously that hyperphosphorylation of Rb in the late G_1 phase of the cell cycle, presumably by CDKs, correlates with decreased binding of E2F1 to Rb and derepression and/or transactivation of genes involved in G_1/S cell cycle progression and cell proliferation (30). However phosphorylation of Rb by other kinases, including TG2, may be important in certain circumstance. For example, ERK1/2-induced Rb phosphorylation at Ser^{780} and Ser^{795} rather than CDK-dependent phosphorylation appears to be important in epidermal growth factor-induced proliferation of small intestinal crypt stem cells (46). In FRTL-5 cells, cAMP-induced proliferation involves both activation of PKA and phosphorylation of Rb (49). It is possible that in this latter situation, phosphorylation of Rb by TG2 could be important. The differences in cell cycle progression in MEF$^{tg2+/+}$ and MEF$^{tg2-/-}$ cells reported here would be consistent with a role of TG2 modulating Rb phosphorylation. Translocation of TG2 to the nucleus under circumstances in which TG2 kinase activity predominates and its transamidating activity is suppressed (*e.g.* high ATP, low Ca^{2+} levels) could favor cell proliferation and protect against apoptosis by enhancing the Rb phosphorylation state. Clearly the regulation of E2F activity by Rb is complex; multiple regulatory pathways, in addition to the cyclin-dependent kinases, are likely to be involved. Under certain circumstances E2F1 can also activate the apoptotic pathway (50), a situation in which CDKs are unlikely to be important. This latter phenomenon may be explained by the observation that E2F1 can induce expression of ARF, a protein that interacts physically with Mdm2 and possibly p53 itself (51), preventing its degradation and allowing for accumulation of transcriptionally active p53, which can induce apoptosis (31). In this regard, it is notable that p53 is also a substrate for TG2 kinase activity (51).

In summary we have demonstrated that TG2 is able to phosphorylate Rb. Furthermore, we have also demonstrated that ATP and Ca^{2+} concentrations and the TG2 phosphorylation state serve as switching mechanisms to change the functional activity of TG2 directed against this substrates. Obviously multiple factors are involved in the regulation of apoptosis and cell cycle progression. To date the emphasis has been on the role of the TG2 transamidating activity in apoptosis; however, our data suggest that its recently discovered kinase activity may be equally important, under certain circumstances, in regulating

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the cell cycle progression. The development of agents that specifically inhibit the kinase activity of TG2 and TG2 mutants that lack kinase activity will provide potentially useful tools for further investigation of the role of this multifunctional protein in the cell cycle and apoptosis.

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