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Phosphorylation of Histones by Tissue Transglutaminase*

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Tissue transglutaminase 2 (TG2) has recently been shown to have intrinsic serine/threonine kinase activity. Since histones are known to be cross-linked by TG2, we investigated whether histones are also substrates for TG2 kinase activity. TG2 was able to phosphorylate H1, H2A, H2B, H3, and H4 histones *in vitro*. Using peptide substrates and phosphospecific antibodies we demonstrated that TG2 phosphorylated Ser^{10} in H3 and that this phosphorylation was reduced by acetylation, whereas phosphorylation of Ser^{10} by TG2 enhanced acetylation. Furthermore we demonstrated that exogenous TG2 phosphorylated H1 and H3 in nucleosome preparations. We examined the abundance of TG2 in DNA-associated proteins from MCF-7 cells treated with phorbol ester (TPA) and 17β-estradiol (E2). TG2 abundance was significantly reduced in E2-treated cells and enhanced in TPA-treated cells. In summary we have demonstrated that TG2 is able to phosphorylate purified histone proteins, and H3 and H1 in chromatin preparations, and it is associated with chromatin in breast cancer cells. These studies suggest a novel role for TG2 in the regulation of chromatin structure and function.

Tissue transglutaminase 2 (TG2)[4] is a ubiquitously expressed, calcium-dependent trans-amidating acyltransferase that cross-links proteins resulting in polymerization (1). In addition, it has other less characterized functions including as a protein-disulfide isomerase (2), as a G protein-coupled membrane receptor (3) and as a serine/threonine kinase (4). It is localized to many compartments in the cell including the cell membrane, the cytoplasm, and the nucleus (1). TG2 is involved in a multitude of cellular process of which the best studied is apoptosis where it is responsible for the formation of apoptotic bodies.

TG2 is translocated to the nucleus by a mechanism that involves importin-α3 (5, 6), where it can cross-link histones (7, 8). Cross-linking of the core histone subunits, H2A and H2B, by TG2 appears to involve glutamine and lysine residues at COOH-terminal domain (9). Histone H1, has also been shown to be a substrate for TG2 (9). Histones bind in a sequence-independent manner to DNA to form chromatin. They are subject to extensive post-translational modification that appears to be important in regulating chromatin function. For example, the amino-terminal tails of histones can be both phosphorylated and acetylated and these modifications are thought to regulate chromatin structure to facilitate transcription, DNA replication, mitosis, and DNA repair.

Histone cross-linking by TG2 may be responsible for the changes in DNA function such as transcription and replication (11), although the exact molecular mechanisms responsible have not been elucidated, and many apparently contradictory data exist. For example, it appears that TG2 can only cross-link histones when released from the nucleosome. Since cross-linking of histone by TG2 does not occur when the histones were organized in nucleosome (9), it is not immediately obvious how this cross-linking activity of TG2 could modulate gene transcription.

We have recently demonstrated that TG2, in addition to its ability to cross-link proteins, also has intrinsic kinase activity (4). The core histones, H2A, H2B, H3, and H4, and histone H1 are known to be phosphorylated, and it has been suggested that phosphorylation of certain histones, for example H3 by kinases such as mitogen- and stress-induced kinase, MSK1, promotes gene transcription (12). Furthermore, rapid changes in histone phosphorylation patterns have also been documented after induction of apoptosis although the enzymes responsible remain unknown (13). TG2 is activated by a variety of agents that induce apoptosis (14, 15), and TG2 null cells are relatively resistant to apoptotic stimuli (16). However, TG2 can also protect against apoptosis under some circumstances (17, 18). It has been assumed that it is the TG2 cross-linking activity that is important in apoptosis, although there are few experimental data that directly test this hypothesis. The different functional roles of TG2 may be related to activation state of TG2, coenzymes, or its different localization within the cell (19). In this report we investigated whether histones are substrates for TG2 kinase activity.

MATERIALS AND METHODS

Reagents—MCF-7 and COS-1 cells were obtained from the American Type Tissue Collection (Manassas, VA). Cell culture reagents were from Invitrogen (Burlington, Ontario, Canada). Recombinant human TG2, expressed in insect cells, was purchased from Roboscreen (Leipzig, Germany). All other reagents, unless otherwise stated, were obtained from Sigma Canada (Oakville, Ontario, Canada). Protein molecular weight markers were from Bio-Rad (Mississauga, Ontario, Canada).

In Vitro Phosphorylation Assay—Recombinant histone proteins or NH_2-terminal peptides (obtained from Upstate Biotechnology) were incubated separately with 0.25 μg of human recombinant TG2 in kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM magnesium, 50 μM ATP, and [γ-^{32}P]ATP (60 μCi/ml)) for 30 min at 30 °C. To study the effect of phosphorylation on acetylation only cold ATP was used, and at the end of the phosphorylation protocol, p300, histone acetyltransferase (HAT) domain, 0.4 μg, (Upstate Biotechnology) and 90 pmol of [^{3}H]acetyl-CoA (1–10 Ci/mmol, PerkinElmer Life Sciences) were added to the tubes and further incubated for 30–45 min in a final 40 μl volume. Reaction was stopped by addition of SDS-PAGE sample buffer. Samples were boiled for 5 min and analyzed on SDS-PAGE. Subsequently, gels were dried and processed for autoradiography. In some cases proteins were transferred to nitrocellulose membranes and analyzed by Western

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[4] The abbreviations used are: TG2, tissue transglutaminase 2; TPA, phorbol ester; E2, 17β-estradiol; HAT, histone acetyltransferase; HRP, horseradish peroxidase; DMEM, Dulbecco's modified Eagle's medium.

blot. For quantification phosphorylated proteins and peptides were separated on SDS-PAGE, and gels were stained with Coomassie Blue. Bands corresponding to phosphorylated proteins and peptides were excised and the radioactivity measured in a β counter. [^{32}P]Phosphate incorporated was expressed per picomole of substrate.

In Vitro Protein Acetylation Assay—To study the effect of acetylation on phosphorylation, 500 ng of recombinant protein or NH_2-terminal peptides of core histones were incubated with recombinant p300, HAT domain, 0.4 μg, in reaction buffer (50 mM Tris-HCl, pH 7.5, containing 50 μM acetyl-CoA) for 30–45 min. At the end of reaction, 0.25 μg of TG2 and 60 μCi/ml [γ-^{32}P]ATP along with 10 mM $MgCl_2$, 50 μM ATP were added to the tubes and further incubated for 30 min at 30 °C. The reaction was stopped by addition of SDS-PAGE sample buffer, and the samples were boiled for 5 min and analyzed by SDS-PAGE. Subsequently, gels were dried and processed for autoradiography or transferred to nitrocellulose membranes and analyzed by Western blot. For autoradiography of the acetylated proteins, gels were treated with EN^{3}HANCE (PerkinElmer Life Sciences) as per the manufacturer's protocol. For quantification of radioactivity bands corresponding proteins and peptides were excised, and the radioactivity was measured in a β counter.

Western Blotting—For Western blotting, proteins were separated on 16% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 5% milk and then incubated with the respective primary antibodies for 1 h at room temperature. For detection of phosphorylation of a specific amino acid membranes were incubated with phosphospecific primary antibodies (1:500, Upstate Biotechnology) 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 (HRP)-conjugate secondary antibodies for 1 h at room temperature. For blotting of the biotinylated histone peptides, after blocking in 5% milk, membranes were incubated with streptavidin-HRP conjugate (1:10,000 dilutions) for 1 h at room temperature. After washing membranes were analyzed with ECL reagent from Amersham Biosciences.

Streptavidin-Agarose Pull-down Assay—COS-1 cells were cultured in DMEM with 10% fetal calf serum. Biotinylated NH_2-terminal histone peptides, 2 μg (Upstate Biotechnology) were incubated with 20 μl of streptavidin-agarose (Sigma) in 500 μl of Tris-HCl buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40) for 1 h at 4 °C. At the end of incubation, agarose beads were washed three times in Tris-HCl buffer as described above. 500 μl of COS-1 cell lysate in Tris-HCl buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.3 μM aprotinin, 0.5% Nonidet P-40) were added to each tube and incubated for 2 h at 4 °C with constant stirring. The tubes were then centrifuged for 30 s at 7000 rpm, and the pellets were washed five times in Tris-buffered saline. Pellets were boiled for 8 min in 30 μl of Laemmli buffer and analyzed by SDS-PAGE followed by Western blot using goat polyclonal anti-transglutaminase (1:1000) and streptavidin-HRP (1:7500) as described above. Guinea pig myelin basic protein (Sigma) was biotinylated as described previously (4) and included as a control for nonspecific interaction of TG2 with basic proteins.

Cell Culture and Treatments—Hormone-dependent, estrogen receptor-positive MCF-7 human breast carcinoma cells were maintained in complete culture medium containing DMEM (Invitrogen) supplemented with 5% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a 37 °C humidified incubator with 5% CO_2. Once 60–70% confluence was reached, cells were estrogen- and serum-depleted in phenol red-free DMEM (Sigma) containing penicillin (100 units/ml), streptomycin (100 μg/ml), 5% glucose, 0.1% bovine serum albumin (Sigma), and apotransferrin (10 μg/ml) to drive the majority of the cell population in G_0/G_1. Cells were either left untreated or treated with 10 nM E2 for 45 min or with 100 nM TPA for 30 min.

Nucleosome Preparation and Histone Isolation—Chicken immature erythrocyte salt-soluble chromatin S150 was prepared as described previously (20). The salt-soluble chromatin fragments are enriched in acetylated core histones and will remain soluble in the kinase buffer, which contains 10 mM $MgCl_2$. Histones were isolated from MCF-7 cell pellets by 0.4 N H_2SO_4 acid extraction (21). To determine the phosphorylation of specific amino acids by TG2 in H3 present in the nucleosome, 50 μl of agarose-alkaline phosphatase conjugate (Sigma) was washed three times in incubation buffer (50 mM Tris-HCl, 120 mM NaCl, pH 8.0) and finally resuspended in 50 μl of the same buffer. Five micrograms of nucleosomes prepared from serum-starved MCF-7 cells were incubated with 40 μl of the enzyme for 1 h at 37 °C. At the end of the incubation, the immobilized enzyme was removed by centrifugation. The supernatant was collected, divided into three tubes, and subsequently processed for TG2-induced phosphorylation in the presence of NaF (100 mM) and Na_3VO_4 (100 mM). Phosphorylated proteins were separated on SDS-PAGE, transferred to the nitrocellulose membranes, and processed for Western immunoblot using phospho-specific antibodies.

Isolation of DNA-associated Proteins by Formaldehyde Cross-linking—After treatments indicated above, $\sim 1 \times 10^9$ cells MCF-7 cells were incubated with 1% formaldehyde for 10 min at room temperature as described previously (22). Reactions were quenched with the addition of 125 mM glycine in phosphate-buffered saline. After washing, cells were collected and resuspended in lysis buffer (5 M urea, 2 M guanidine hydrochloride, 2 M NaCl, and 0.2 M potassium phosphate buffer, pH 7.5). Sonication was carried out to solubilize chromatin and associated proteins, and the cell lysate was prepared after centrifugation. The cell lysate was then incubated with hydroxyapatite (Bio-Rad) (1 g of hydroxyapatite/80A_{260}). After three washes with ice-cold lysis buffer, the proteins cross-linked to DNA were reversed at 65 °C for over 6 h, dialyzed, and lyophilized.

RESULTS

TG2 Phosphorylates Histones—All four core histones and H1 were phosphorylated by TG2 under *in vitro* conditions (Fig. 1, *A* and *C*). Histone H2B was more highly phosphorylated by TG2 than H2A (Fig. 1*A* and Table 1). When equal molar amounts of H2A protein and the H2A$^{1-21}$ fragment were analyzed the level of label incorporation was 4-fold greater in the protein compared with the NH_2-terminal peptide suggesting that additional sites of the phosphate incorporation occurred throughout the H2A molecule (Fig. 1*A* and Table 1). In contrast, the H2B$^{1-21}$ fragment was only lightly radiolabeled compared with the histone H2B protein indicating that the majority of the labeling of histone H2B was occurring elsewhere in the protein. The histone H3$^{1-21}$ fragment was phosphorylated by TG2, whereas the H3$^{21-44}$ peptide was not. The majority of the TG2-induced phosphorylation of H4 occurred in the H4$^{1-21}$ domain (Table 1). In the absence of TG2 none of the histone peptides were radiolabeled indicating that the incorporation of the phosphate group was due to TG2 and not a result of autophosphorylation or a contaminating kinase (Fig. 1, *B* and *C*). Although the histone H3$^{1-21}$ fragment was phosphorylated there was evidence that TG2 phosphorylated additional residues elsewhere in the H3 protein (Fig. 1*B*). Approximately one-third of the TG2-induced ^{32}P incorporation into H3 occurred in the H3$^{1-21}$ domain (Table 1). A comparison of the histone fragments H2A$^{1-21}$, H2B$^{1-21}$, and H3$^{1-21}$ peptides is also shown in Fig. 1*D*. The H2A$^{1-21}$ peptide was slightly more strongly labeled than

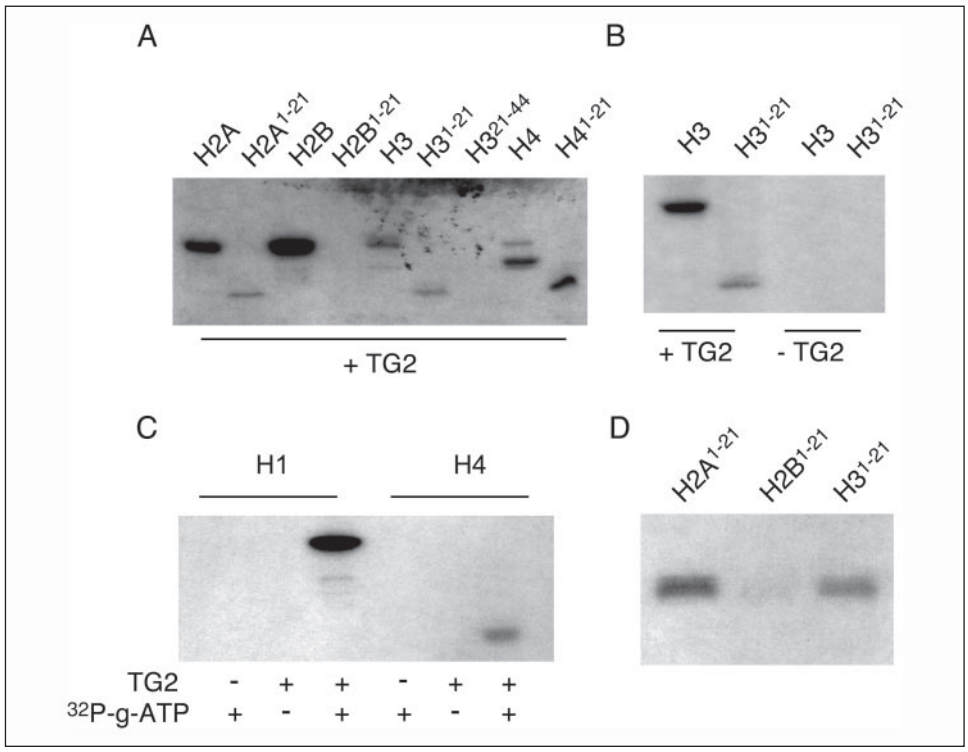

FIGURE 1. **TG2 phosphorylates core histones.** In *A*, equimolar amounts of histones or peptides were incubated with TG2 and [γ-^{32}P]ATP and subsequently analyzed by SDS-PAGE and autoradiography. Incorporation was also quantified (Table 1). In *B*, equimolar amounts of H3 protein and H3$^{1-21}$ peptide were incubated with TG2. In *C*, H1 and H4 proteins were incubated in the presence or absence of TG2 and [γ-^{32}P]ATP as indicated and subsequently analyzed for radiolabeling as described above. In *D*, equimolar amounts of H2A$^{1-21}$, H2B$^{1-21}$, and H3$^{1-21}$ peptides were incubated with TG2 and ^{32}P-γ-ATP.

TABLE 1
TG2 catalyzed incorporation of phosphate into core histones and histone N-terminal peptides

Substrate	[^{32}P]Phosphate incorporated
	pmol/pmol of substrate
H1 protein	1.42
H2A protein	1.31
H2A$^{1-21}$ peptide	0.32
H2B protein	1.50
H2B$^{1-21}$ peptide	0.03
H3 protein	0.94
H3$^{1-21}$ peptide	0.27
H3$^{21-44}$ peptide	0.01
H4 protein	1.08
H4 $^{1-21}$ peptide	0.91
H1 in nucleosome	0.29
H2A in nucleosome	Not detected
H2B in nucleosome	Not detected
H3 in nucleosome	1.26
H4 in nucleosome	Not detected

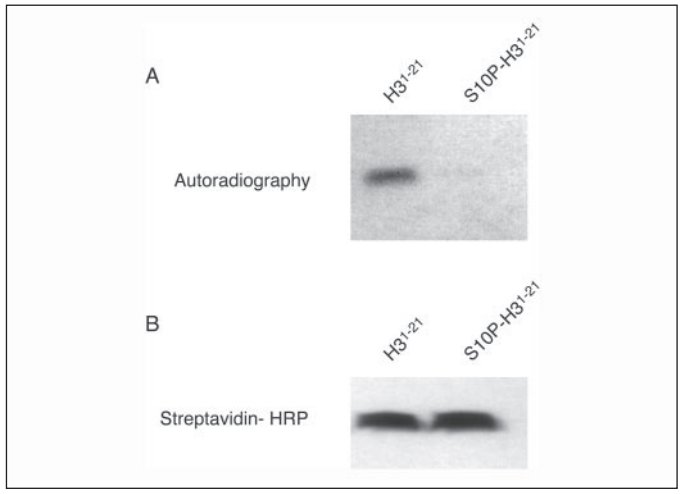

FIGURE 2. **TG2 phosphorylates H3 at Ser^{10}.** In *A*, equimolar amounts of H3$^{1-21}$ and S10P-H3$^{1-21}$ peptides were incubated with TG2 and [γ-^{32}P]ATP and subsequently analyzed by SDS-PAGE and autoradiography. When quantified, incorporation of ^{32}P was ~0.3 pmol/pmol of H3$^{1-21}$ peptide. Incorporation of ^{32}P into the S10P-H3$^{1-21}$ peptide was unmeasurable. In *B*, the same membrane was probed with streptoavidin-HRP to demonstrate equal loading.

H3$^{1-21}$, whereas the H2B$^{1-21}$ peptide showed the lowest level of phosphate incorporation. When equivalent amounts of H3$^{1-21}$ peptide and the S10P-H3$^{1-21}$ peptide, where the serine at position 10 was already phosphorylated, were incubated with TG2 and [γ-^{32}P]ATP very little labeling of the S10P-H3$^{1-21}$ peptide was observed (Fig. 2*A*). The presence of the biotin label at the COOH-terminal end of these peptides allowed for the blot to be probed with streptavidin-HRP to demonstrate equivalent loading on the gel (Fig. 2*B*).

To identify other potential sites of TG2-mediated phosphorylation in H3, we examine another H3 peptide, H3$^{21-44}$, that contains Ser^{28} another residue where phosphorylation is thought to be functionally important. H3$^{21-44}$ was not phosphorylated by TG2 (Fig. 1*A*); however, when H3 was used as a substrate phosphorylation of Ser^{28} was detected with phosphospecific antibody (Fig. 3).

Effects of Acetylation on TG2-induced Phosphorylation—The effect of p300 HAT-induced acetylation of the histone peptides on TG2 phosphorylation was examined. Acetylation had no effect on TG2 phosphorylation of the H2A$^{1-21}$ and H2B$^{1-21}$ peptides but reduced radiolabeled phosphate incorporation into the H3$^{1-21}$ (Fig. 4*A* and Table 2). We next examined the effect of TG2-induced phosphorylation on HAT-induced labeling of the histone peptides using [^{3}H]acetyl-CoA. The H2A$^{1-21}$, H3$^{1-21}$, and the S10P-H3$^{1-21}$ peptides were acetylated (Fig. 4*B*). In contrast little incorporation of radiolabel was seen with the H2B$^{1-21}$ peptide (Table 2). TG2-induced phosphorylation enhanced acetylation of the H3$^{1-21}$ peptide by ~2-fold (Table 2) but, as anticipated, had no effect on the acetylation of the S10P-H3$^{1-21}$ peptide.

TG2 Phosphorylates Histone H3 in Nucleosomes—Salt-soluble chromatin fragments from avian erythrocytes were incubated with TG2 in kinase buffer containing [γ-^{32}P]ATP. The major histone bands were

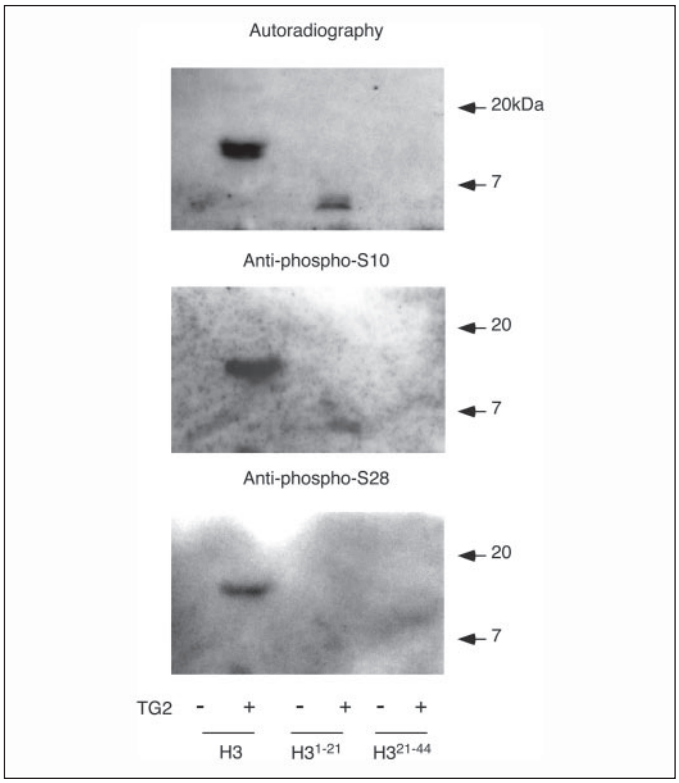

FIGURE 3. **TG2 phosphorylates H3 at Ser[10] (*S10*) and Ser[28] (*S28*).** Equimolar amounts of recombinant H3 protein or $H3^{1-21}$ and $H3^{21-44}$ peptides were phosphorylated by TG2 in the presence of [γ-^{32}P]ATP or ATP and subsequently analyzed by SDS-PAGE and autoradiography. Simultaneously run gels were analyzed by immunoblotting using antiphospho-Ser[10] and antiphospho-Ser[28] antibody.

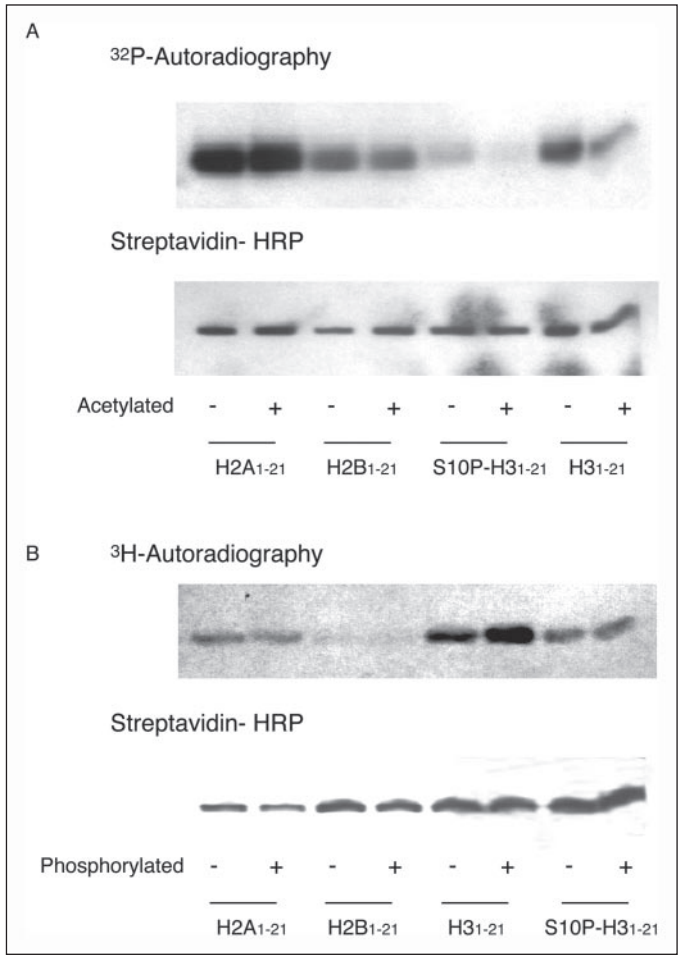

FIGURE 4. **The interaction of acetylation and TG2-induced phosphorylation.** In *A* TG2-induced phosphorylation of non-acetylated and HAT-acetylated H2 and H3 peptides was examined. In *B*, equimolar amounts of the peptides were initially incubated with or without TG2 in the presence of unlabeled ATP prior to incubation with HAT and [^{3}H]acetyl-CoA. In each case the same filters were probed with streptavidin-HRP to demonstrate equal loading. Similar data were obtained in a separate experiment and has been quantified in Table 2.

separated on a 16% SDS-PAGE and identified by Coomassie Blue staining. The individual histone bands were excised and re-run on a similar gel transferred to nitrocellulose paper and identified by autoradiography and Ponceau S staining (Fig. 5*A*). Under these conditions histone H2A, H2B, and H4 were not phosphorylated (Table 1). However, histone H3 was intensely radiolabeled. A small amount of phosphate was also incorporated into histone H1 (Fig. 5*A*). In the absence of TG2 no histone phosphorylation was observed indicating that the phosphorylation of H3 and H1 was due to the added TG2 and not due to a contaminating kinase in the nucleosome preparation (Fig. 5*B*).

To determine which residues in H3 were phosphorylated in the nucleosome preparation we initially dephosphorylated the nucleosome preparation using immobilized alkaline phosphophase prior to incubation with TG2. Under these condition Ser[10] and Ser[28] were identified using phosphospecific antibodies (Fig. 6).

TG2 Interacts with Histones—Biotinylated histone peptides were used to examine the interaction of TG2 with histones. COS-1 cell extracts that contain large amounts of TG2 were incubated with H2A, H2B, H3, and H4 NH_2-terminal peptides. In this assay H3 peptide was the most efficient at pulling down TG2. H4 peptide was the least efficient (Fig. 7*a*). This was particularly apparent when the gel was reprobed with streptavidin-HRP, demonstrating that more H2A and H2B peptides were present in the pulled downs compared with H3 and H4 peptides (Fig. 7*B*). No interaction was apparent with myelin basic protein.

TG2 Is Associated with Chromatin in MCF-7 Cells—DNA associated proteins from control and E2- and TPA-treated MCF-7 cells were prepared by the formaldehyde cross-linking technique and analyzed by SDS-PAGE and immunoblotting. Probing with anti-H3 antibody indi-

TABLE 2

The effect of HAT induced acetylation on TG2 mediated phosphorylation of histone N-terminal peptides and vice versa

NA, non-acetylated; A, acetylated; NP, non-phosphorylated; P, phosphorylated.

Effect of acetylation on phosphorylation	
Substrate	**[^{32}P]Phosphate incorporated**
	pmol/pmol of substrate
$H2A^{1-21}$ (NA)	0.32
$H2A^{1-21}$ (A)	0.34
$H2B^{1-21}$ (NA)	0.12
$H2B^{1-21}$ (A)	0.10
$H3^{1-21}$ (NA)	0.32
$H3^{1-21}$ (A)	0.05
S10P $H3^{1-21}$ (NA)	0.02
S10P $H3^{1-21}$ (A)	0.01
Effect of phosphorylation on acetylation	
	[^{3}H]Acetate incorporated
	pmol/pmol of substrate
$H2A^{1-21}$ (NP)	0.36
$H2A^{1-21}$ (P)	0.33
$H2B^{1-21}$ (NP)	0.01
$H2B^{1-21}$ (P)	0.02
$H3^{1-21}$ (NP)	0.56
$H3^{1-21}$ (P)	1.21
S10P $H3^{1-21}$ (NP)	0.29
S10P $H3^{1-21}$ (P)	0.31

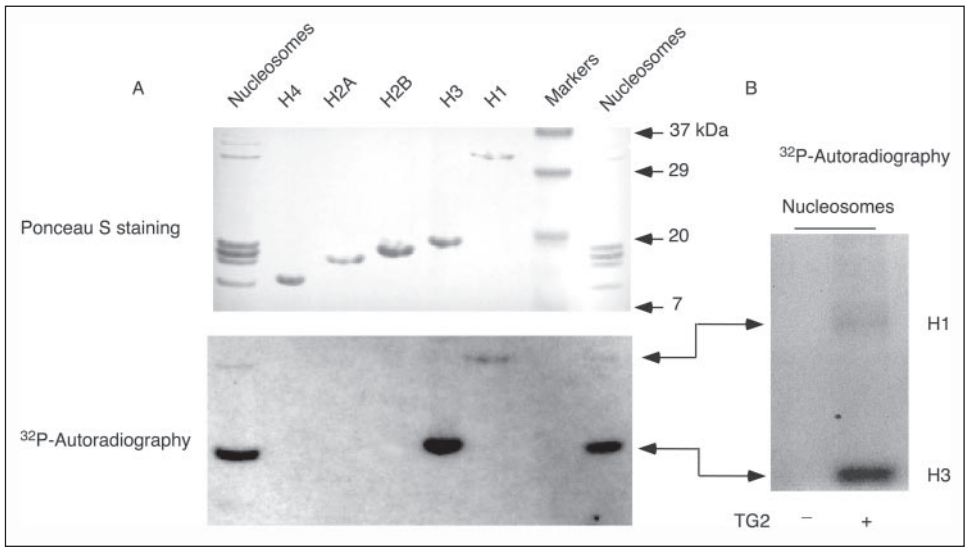

FIGURE 5. **TG2 phosphorylates H1 and H3 in nucleosomes.** In *A*, nucleosomes were incubated with TG2 and [γ-^{32}P]ATP. Individual histones were separated on a 16% SDS-PAGE gel, excised, and re-analyzed. The position of the molecular weight markers is indicated. In *B*, nucleosomes were incubated with [γ-^{32}P]ATP in the presence or absence of TG2 as a control for endogenous kinase activity. These data were replicated on a separate occasion.

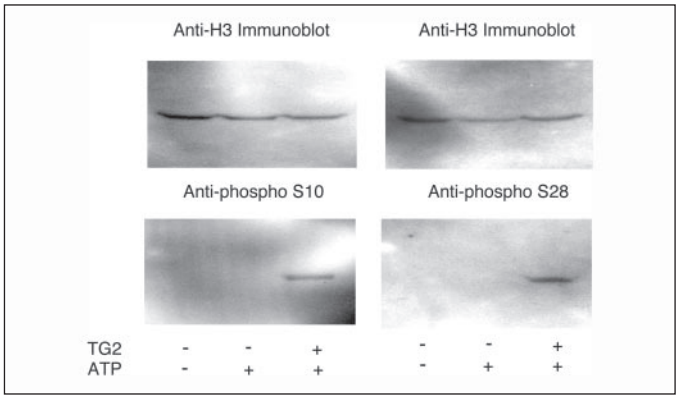

FIGURE 6. **TG2 phosphorylates Ser^{10} (*S10*) and Ser^{28} (*S28*) in H3 in nucleosomes.** Nucleosomes were prepared from MCF-7 cell nuclei and treated with immobilized alkaline phosphatase to reduce endogenous phosphorylation prior to incubation with TG2 and ATP. Samples were analyzed by SDS-PAGE and immunoblotting.

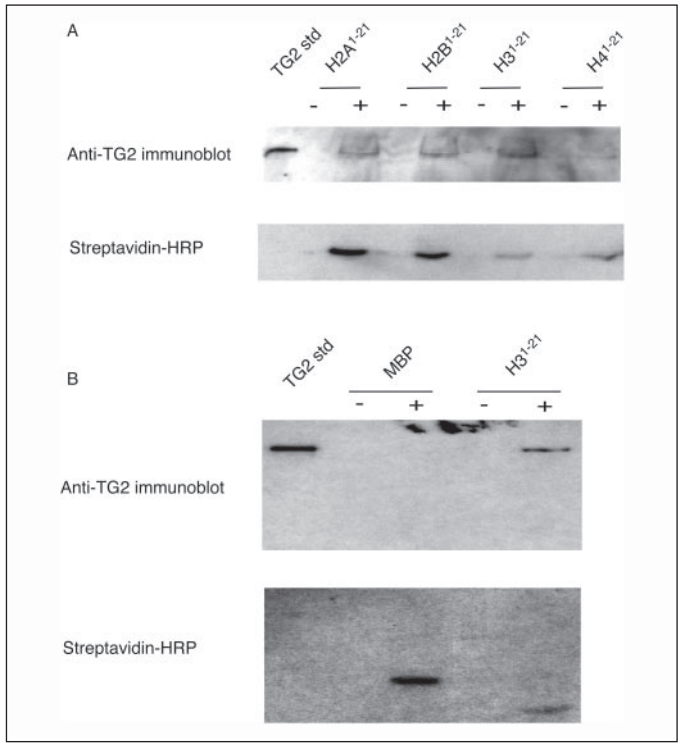

FIGURE 7. **TG2 binds to biotinylated histone peptides.** In *A*, whole cell extract from COS-1 cells was incubated with the various peptides. After a streptavidin-agarose pull-down the pellets were analyzed by SDS-PAGE and immunoblotting with anti-TG2 antisera (*upper panel*). The filter was subsequently re-probed with streptavidin-HRP (*lower panel*). A TG2 standard is included on the gel as a positive control. Similar data were obtained in a separate experiment. In *B*, as a control for nonspecific interaction of TG2 with basic proteins, biotinylated guinea pig myelin basic protein (*MBP*) was added to COS-1 cells and extracted and analyzed as above.

cated similar abundance in all three experimental conditions (Fig. 8*A*). However, TG2 abundance was significantly reduced in E2-treated cells and enhanced in TPA-treated cells (Fig. 8*B*).

DISCUSSION

Here we extend our previous observations that TG2 has intrinsic kinase activity (4) and demonstrate that histones, known substrates for the cross-linking activity of TG2 (8, 9), are also substrates for TG2 kinase activity. We have previously shown that the K_m and V_{max} for the TG2 kinase reaction are comparable with other kinases (4) and thus are likely to physiologically relevant *in vivo*. The recombinant TG2 is expressed in insect cells and is greater than 90% pure. The kinase activity is not due to combination, since it can be immunoprecipitated by anti-TG2 antibody (4), kinase activity is also apparent with *Escherichia coli* expressed human TG2 and the kinase activity co-migrates with TG2 in an *in gel* kinase assay (data not shown). Under *in vitro* conditions TG2 was able to phosphorylate all four histones, and this phosphorylation occurred at functionally important residues such as Ser^{10} and Ser^{28} in H3 and serines resides in the NH_2-terminal region of H2A. Furthermore, we demonstrate that TG2 is able to phosphorylate H3 and H1 in nucleosome preparations.

Approximately 30% of the TG2-induced phosphorylation of H3 occurred at Ser^{10}, a site that has previously been shown to be important in expression of immediately early genes (22) and crucial for chromosome condensation and cell cycle progression (23). Several kinases have been identified that have the potential to phosphorylate H3 at Ser^{10} *in vivo* (24), and our data suggest that TG2 can function in this manner *in vitro*. Acetylation at Lys^{14} and possibly Lys^{9} in H3 appear to be coupled in some way to phosphorylation at Ser^{10} *in vivo* (23, 25). Consistent with these previous reports we demonstrated that acetylation of the H3$^{1-21}$ peptide *in vitro* reduced TG2-induced phosphorylation at Ser^{10}, whereas TG-2 induced phosphorylation of the H3$^{1-21}$ peptide

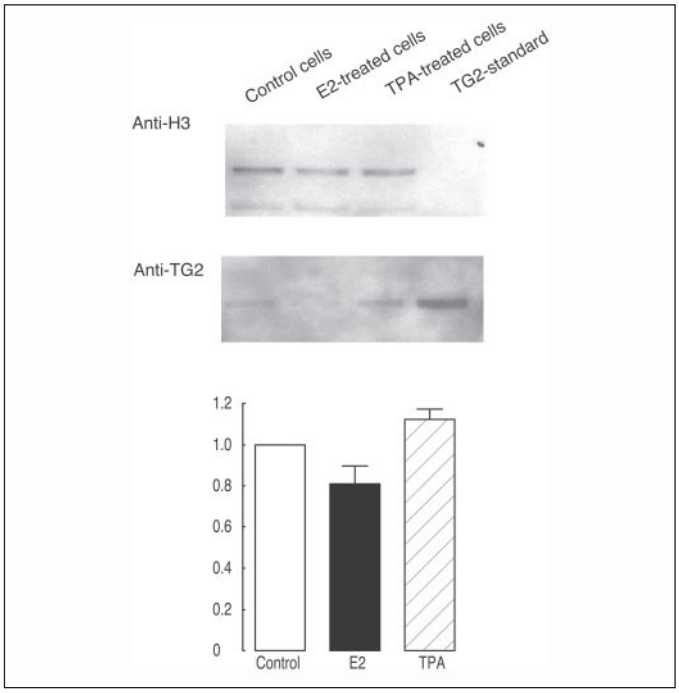

FIGURE 8. **Detection of TG2 in DNA-associated proteins from MCF-7 cells.** DNA-associated proteins were prepared from MCF-7 cells treated with E2 or TPA using the formaldehyde cross-linking technique. The presence of TG2 and H3 were detected by immunoblotting. In the *lower panel* data from four separate experiments were quantified by densitometry.

enhanced acetylation. Additional phosphorylation of H3 occurred at Ser^{28}, which has also been shown to be functionally important in the regulation of gene expression (22). The $H3^{21-44}$ peptide did not appear to be a good substrate for TG2 kinase activity *in vitro* possibly because of secondary structure. Consequently it was not possible to determine what percentage of the total TG2-induced ^{32}P incorporation occurred at this site.

The presence of TG2 in the nucleus has been reported in various cell lines (9, 26–28). In the nucleus TG2 appears to serve a variety of functions. It is involved both in histone cross-linking and chromatin condensation into apoptotic bodies (29). It is also involved in DNA degradation and may have a previously unrecognized DNA hydrolytic activity (30). In addition TG2 modulates expression of a variety of transcription factors such as NFκB (27) and Sp1 (31) and thereby indirectly modulates transcription of a multitude of genes. There has been a tendency to attribute the majority of TG2 actions to its known crosslinking activity. For example, Han and Park (31) demonstrated that purified TG2 can enhance the binding activity of Sp1 to the target DNA sequence by gel electrophoretic mobility shift assay and when co-transfected increased the activity of the human p21WAF1 promoter containing six Sp1 binding sites. They suggest that this effect was due to the ability of TG2 to cross-link Sp1, but this point was not directly addressed, and an alternative explanation is that TG2 could directly or indirectly alter the phosphorylation state Sp1 that has previously been shown to be important in Sp1 transcriptional activity (32). It is difficult to directly determine which of the various activities of TG2 are involved in the numerous physiological functions that TG2 has been implicated in. While the TG2 mutants have been described (33, 34), it has not been determined whether these mutants, which lack cross-linking activity, also lack other TG2 functions such as kinase activity, protein-disulfide isomerase activity, DNA hydrolytic activity, etc. Experiments where TG2 expression is increased by co-transfection or silenced by knockdown techniques (35, 36) are also difficult to interpret, since these perturbations may also affect activities of TG2 other than its crosslinking activity. Similarly, interpretations of experiments using monodansylcadaverine, an inhibitor of TG2 transamidation, are problematic since this agent also inhibits TG2 kinase activity (4).

The functional activity of TG2 appears to be modulated by divalent cations and nucleotides (37–40). The cross-linking activity of TG2 requires Ca^{2+}, whereas the DNA hydrolytic activity appears to be dependent on Mg^{2+} rather than Ca^{2+} (30). We have reported previously that TG2 kinase activity is abrogated by high Ca^{2+} concentrations and enhanced by ATP (4) whereas the cross-linking activity of TG2 is Ca^{2+}-dependent and inhibited by ATP (1). Thus the possibility exists for the functional activities of TG2 to be modulated by the local milieu.

Consistent with previous reports of immunolocalization of TG2 to the nucleus (19), we clearly show that TG2 can phosphorylate H3 and to a lesser extent H1 in chromatin and that TG2 is associated with chromatin *in situ* in human breast cancer cells. We also demonstrated in pull-down experiments that TG2 has an affinity for histone proteins. The association of TG2 with chromatin was reduced by E2, a treatment known to enhance cell proliferation in MCF-7 cells, and enhanced by TPA, which inhibits cell proliferation under these conditions.

In summary we have clearly demonstrated that TG2 is able to phosphorylate histones and is associated with chromatin in cells. Although it remains unclear the relative importance of TG2 kinase induced histone phosphorylation in modulation of gene transcription or apoptosis, our observations suggests the numerous previous reports of TG2 modulation of transcription of various signaling molecules need to be reassessed in light of TG2 kinase activity. Further investigations with TG2 mutants that lack specific functional activities are required to determine the relative importance of TG2 transamidation, TG2-induced phosphorylation, and other activities of this multifunction protein.

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