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The p53 oncoprotein is a substrate for tissue transglutaminase kinase activity ☆

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Abstract

Increased expression and activity of the ubiquitous enzyme, tissue transglutaminase (TG2), is consistently seen in a variety of models of apoptosis. The p53 oncoprotein is also involved in apoptosis. Here we investigated the interaction of TG2 with p53 and show that the p53 is a substrate for the recently identified serine/threonine kinase activity of TG2. Phosphospecific antibodies indicated that TG2 phosphorylated p53 at Ser^{15} and Ser^{20}, residues that are critically important in the interaction of p53 with Mdm2. The TG2-induced phosphorylation was abrogated by high Ca^{2+} concentrations and inhibited by cystamine, a known inhibitor of TG2 cross-linking activity. Furthermore, we demonstrate that TG2-induced phosphorylation of p53 reduces the ability of p53 to interact with Mdm2. Although TG2 cross-linking activity has been clearly implicated in apoptosis, our observations reported here suggest TG2 modification of p53 could be an additional mechanism whereby TG2 could facilitate apoptosis.

Keywords: Transglutaminase; p53; Mdm2; Apoptosis; Serine/threonine kinase

TG2 is a ubiquitous enzyme that is part of a group of evolutionary conserved proteins that mediate post-translational protein modification and protein–protein interactions [1]. The best characterized function of TG2 is as a calcium-dependent transamidating acyltransferase 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. However, TG2 has additional activities and it has also been reported to be a protein disulfide isomerase [2], and a G protein coupled membrane receptor [3]. TG2 has been shown to have a role in transmitting signals from classical seven-transmembrane helix G-coupled receptors such as the α_{1B}-adrenergic receptor [4]. We recently identified TG2 as a serine/threonine kinase present in human breast cancer cell membranes responsible for the phosphorylation of insulin-like growth factor (IGF) binding protein-3 (IGFBP-3) [5], an IGF-binding protein that also has IGF-independent pro-apoptotic effects in various cell types [6]. The K_m and V_{max} for TG2-mediated phosphorylation of the IGFBP-3 substrate [5] were in the physiological range and similar to that described for other kinase [7,8].

Activation of TG2 gene expression is an early event in the apoptotic process in response to a wide variety of apoptotic stimuli [9–11], while TG2 activity catalyzing the formation of ε–γ glutamyl-lysine cross-links between polypeptide chains and formation of apoptotic bodies is apparent only later in the apoptotic process [12]. 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 [13,14]. However, TG2 expression is detectable in cells prior to induction of apoptosis suggesting that if the cross-linking activity of TG2 is indeed important in the apoptotic process some mechanism must exist to inhibit cross-linking activity under non-apoptotic conditions.

☆ *Abbreviations:* TG2, tissue transglutaminase; IGFBP-3, insulin-like growth factor binding protein-3; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

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The p53 is a short-lived tumor suppressor protein that when activated functions as a transcription factor [15]. The genes induced by p53 include genes involved in cell cycle arrest and DNA repair genes. It appears to be particularly important in the regulation of transition from the G2 to M phase of the cell cycle [16]. Activation of p53, which causes cell cycle arrest and apoptosis, involves destabilization of the p53–Mdm2 interaction [17]. Mdm2 interacts with the N-terminal region of p53 and phosphorylation of serine residues in this region disrupts the interaction with Mdm2 [17].

Although both p53 and TG2 are involved in apoptosis, the potential interaction of these important proteins has not been investigated in the past. Here we report that p53 is a substrate for TG2 kinase activity.

Materials and methods

Antibodies and proteins. IGFBP-3 was obtained from Upstate Biotechnology (Lake Placid, NY). Recombinant his-tagged human TG2, from Roboscreen, Germany, was used for most experiments unless otherwise stated. Recombinant p53 were purchased from Active Motif. Guinea pig liver TG2, cystamine, mouse monoclonal anti-phosphothreonine (clone PTR-8), and anti-phosphoserine (clone PSR-45) antibodies were obtained from Sigma–Aldrich, Canada (Oakville, Ont.). Monoclonal anti-Mdm2, rabbit polyclonal anti-p53 (Ser^{20}), and rabbit polyclonal anti-p53 antibodies were obtained from Santa Cruz Biotechnology. Rabbit polyclonal anti-p53 (Ser^{15}) antibody that recognizes p53 phosphorylated at Ser^{15} was obtained from R&D Systems.

Phosphorylation of p53. Human recombinant p53 (500 ng) was incubated with 0.25 μg human recombinant TG2 in kinase buffer (50 mM Tris–HCl, pH 7.5, 10 mM Mg, 0.1 mM ATP, and 60 μCi/ml [γ-^{32}P]ATP) for 30 min at 30 °C. 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% skimmed milk and incubated with respective primary antibodies for either 1 h at RT or overnight at 4 °C. After incubation, membranes were washed three times in TBST (10 mM Tris, 150 mM NaCl, and 0.05% Tween 20, pH 8.0) and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at RT. After washing, membranes were analyzed with ECL.

Disruption of p53–Mdm2 complexes. MCF-7 cells (from ATCC) were exposed for 12–15 h to 50 μM proteosome inhibitor, *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal, 20 μl of anti-Mdm2 antibody was added to 1 ml MCF-7 cell lysate and incubated for 1 h at 4 °C. Twenty-five microliters 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 PBS and finally suspended in 40 μl buffer (25 mM Hepes, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 100 mM EDTA 0.1%, and Tween 20). Two hundred nanograms of p53 was incubated with 40 μl immunoprecipitate for 1 h in IP buffer at 4 °C. Unbound p53 was removed by washing three times with IP buffer. The precipitate was incubated with 200 ng recombinant TG2 for 30 min in kinase buffer at 4 °C and washed again to remove any p53 that had dissociated from Mdm2. The fraction of p53 that remained bound to the immunoprecipitate was visualized by SDS–PAGE and immunoblotting with anti-p53 antibody.

Results

Both guinea pig liver TG2 and human recombinant TG2 were able to phosphorylate p53 and this phosphorylation was also inhibited by cystamine, a known inhibitor of TG2 cross-linking activity (Fig. 1). IGFBP-3, which has previously been reported to be phosphorylated by TG2 [5], has been included as a positive control for the kinase assay.

The cross-linking activity of TG2 is Ca^{2+}-dependent [9] whereas TG2 phosphorylation of IGFBP-3 is inhibited by high Ca^{2+} concentrations [5]. In Fig. 2, we show that TG2-induced phosphorylation of p53 is also inhibited by Ca^{2+}. These data are consistent with the recent report by Lee et al. [18] that Ca^{2+} inhibits the GTP binding and GTPase activity of TG2. Cross-linking of p53 by TG2 has not been previously reported. However, a comparison of lanes 4 and 7 in Fig. 1 and lane 1 in Fig. 2 suggests that TG2 may indeed cross-link p53 and result in larger MW forms. Although a larger MW form of p53 was apparent in the autoradiogram in the absence of Ca^{2+}, this band was attenuated by increasing Ca^{2+} concentrations suggesting that phosphorylation of this larger p53 complex was also inhibited by Ca^{2+}.

Using phosphoamino acid-specific antibodies we demonstrated that TG2-induced phosphorylation occurred predominantly at the serine residues (Fig. 3A). Antibody to phosphothreonine recognized TG2 phosphorylated IGFBP-3 but did not recognize p53 that had not been incubated with TG2. Although a number of potential phosphoacceptor sites have been identified in p53, the N-terminal region and particularly Ser^{15} and Ser^{20} have

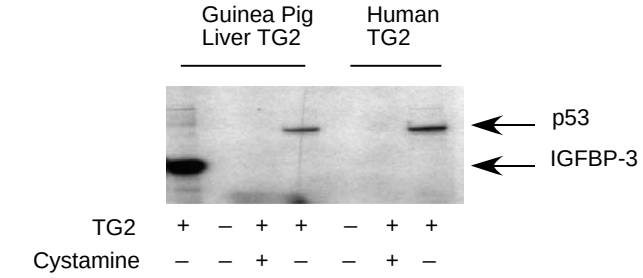

Fig. 1. Tissue transglutaminase (TG2) phosphorylates p53. p53 and [γ-^{32}P]ATP were incubated with guinea pig liver or human recombinant TG2 in the presence or absence of cystamine, an inhibitor of TG2 cross-linking activity, and subsequently analyzed by SDS–PAGE and autoradiography.

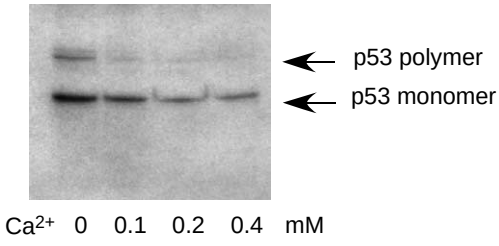

Fig. 2. Transglutaminase kinase activity is inhibited by Ca^{2+}. TG2 was incubated with p53 and [γ-^{32}P]ATP in the presence of increasing concentrations of Ca^{2+}. After analysis by SDS–PAGE, radiolabeled p53 was identified by autoradiography.