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PFT-α inhibits antibody-induced activation of p53 and pro-apoptotic signaling in 1-LN prostate cancer cells

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ABSTRACT

Antibodies against the COOH-terminal domain of cell surface GRP78 induce apoptosis in cancer cell lines via activation of p53 signaling. We now have studied the effects of PFT-α, an inhibitor of p53-mediated apoptotic pathways, on anti-GRP78 antibody-induced activation of p53 and pro-apoptotic signaling in 1-LN prostate cancer cells. Pretreatment of 1-LN cancer cells with this agent significantly inhibited antibody or doxorubicin-induced upregulation of p53. Concomitantly, PFT-α treatment prevented down regulation of ERK1/2 activation by either antibody or doxorubicin. Likewise, PFT-α prevented increases in the pro-apoptotic proteins BAD, BAK, BAX, PUMA, and NOXA as well as activation of caspases-3, -7, and -9. We conclude that antibody-induced apoptosis in prostate cancer cells is mediated predominantly by p53 using the mitochondrial pathway of apoptosis.

Introduction

The molecular chaperone GRP78 (glucose-regulated protein of 78 kDa) is constitutively expressed in eukaryocytes; however, its synthesis is induced by stressful conditions that perturb protein folding and assembly within the ER [1]. This unfolded protein response (UPR) is essential in protecting cells against death. GRP78 is often over expressed in cancers where it mediates tumor growth by enhancing cell proliferation, protecting cells against apoptosis, and promoting tumor angiogenesis and drug resistance [2]. Thus, upregulation of cell surface GRP78 is part of a more aggressive phenotype in a number of tumors [1]. A small pool of newly synthesized GRP78 in association with MTJ1 may escape the KDEL retention mechanism in the ER and relocate to the cell surface particularly in cancer cells [3,4]. GRP78 lacks a transmembrane domain, unlike MTJ1, and both its termini are on the external side of the membrane [3,4]. Cell surface GRP78 functions as a receptor for a number of ligands [1]. Among these the activated form of the plasma proteinase inhibitor α_2-macroglobulin (α_2M^*) is best characterized [3–5]. α_2M^* ligation of cell surface GRP78 in cancer cells triggers mitogenesis and cellular proliferation by activating the PI 3-kinase/Akt, ERK1/2, UPR and NFκB signaling pathways [5]. Serum from patients with various tumors, including prostate and melanoma, often contain high titers of an auto-antibody to GRP78 whose occurrence correlates with a more metastatic phenotype and poor prognosis [6,7]. These antibodies bind to an epitope in the NH_2-terminal domain which is identical to the α_2M^* binding sites and this antibody functions as an agonist [8]. By contrast, antibodies directed against the COOH-terminal domain of GRP78 are antagonists which inhibit cell growth while promoting apoptosis [9].

PFT-α, [2-(2-amino-4,5,6,7-tetrahydrobenzothiol-3-yl)-1-*p*-tolylethanone]-1, inhibits p53 apoptotic signaling by interfering with its nuclear translocation and transcriptional activation of p53 inducible genes [10–12]. In addition to its p53-dependent effects, p53-independent effects of PFT-α have also been reported [10–16]. For example, after DNA damage, it protects against apoptosis in a p53-dependent and p53-independent manner [14]. PFT-α blocks the apoptosome-mediated processing and activation of caspase-9 and -3 in colon carcinoma cells and inhibits induction of p53, p21 and BAX after UV treatment of CS-B cells [15,16]. PFT-α is considered a useful tool to characterize p53-mediated events in a variety of cell types with various apoptosis-inducing agents. In a previous report, we demonstrated that incubation of prostate cancer and melanoma cells with antibody directed against the COOH-terminal domain of GRP78 causes a several fold increase in p53, inhibits DNA synthesis, and induces apoptosis [9]. If antibody-induced cellular death in these cells is mediated by upregulated p53 signaling, then PFT-α should inhibit upregulation of p53 signaling as well as components of the apoptotic pathway. We have tested this hypothesis and we report that pretreatment of prostate cancer cells blunts antibody-induced increase in p53, and pro-apoptotic proteins while preventing caspase activation.

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Materials and methods

Materials. Culture media were from Invitrogen (Carlsbad, CA). α_2M^* was prepared as described previously [5]. Antibody against the COOH-terminal domain of GRP78 (Cat 3 SPA-826 from Stressgen, Victoria, BC) is designated anti-CTD antibody in this report. This antibody, at an IgG concentration of 200 μg/ml, was employed at a 1:200 dilution in these studies. Antibodies against phosphorylated ERK1/2 phosphorylated p53, p53, Bcl-2, phosphorylated MDM2, caspase-3, -7, and -9 were from Cell Signaling Technology, Inc. (Beverly, MA). Antibodies against PUMA, BAD, BAK, BAX, NOXA, were from Santa Cruz Biotechnology. PFT-α and doxorubicin·HCl, Ac-DEVD-AMC and ACLEHD-AMC were from Biomol (Plymouth Meetings, PA). Antibodies against actin were from Sigma (St. Louis, MO).

Cancer cell lines. The highly metastatic human prostate carcinoma cell line 1-LN is derived from the less metastatic PC-3 cells and was a kind gift of Dr. Philip Walther (Duke University Medical Center, Durham, NC) [9]. 1-LN cells are the prototypic cell line demonstrating a high level of GRP78 cell surface expression [17]. In a previous study, however, we observed that all cell lines expressing GRP78 on the cell surface demonstrated a similar response to anti-CTD antibody [8]. In the present study, therefore, we employed the 1-LN line to characterize the ability of PFT-α to mediate p53 signaling in anti-CTD-induced apoptosis. These cells were grown in 6-well plates (5×10^6 cells/well) in RPMI 1640 medium containing 10% FBS, 2 mM glutamine, 12.5 U/ml penicillin, 6.5 μg/ml streptomycin and 10 nM insulin (RPMI-S) in an humidified CO_2 (5%) incubator at 37 °C. At about 90% confluency, the medium was aspirated, the monolayers washed with ice-cold HHBSS and a fresh volume of medium added and these cells used for the experiments described below.

Measurement of PFT-α effects on anti-CTD upregulated p53 signaling, antiapoptotic proteins, and caspase activation by Western blotting. 1-LN cells grown as above were treated with: (1) buffer; (2) anti-CTD antibody (1:200/30 min); (3) doxorubicin (5 μM/1 h); (4) doxorubicin (5 μM/1 h) then anti-CTD (1:200/30 min); or (5) PFT-α (25 μM/2 h) then anti-CTD (1:200/30 min) and incubated as above. The reactions were terminated by aspirating the medium, and adding a volume of lysis buffer A containing 50 mM Tris·HCl (pH 7.5), 120 mM NaCl, 1% v/v NP-40, 20 mN NaF, 1 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate, 1 mM PMSF, 1 mM benzamidine, and leupeptin (20 μg/ml). Cells were lysed on ice for 10 min and lysates collected in microfuge tubes, DNA strands broken by passing the lysates through 27 gauge needle several times, and the tubes centrifuged at 1200 rpm for 5 min at 4 °C. The protein content of the lysates was determined [18] and an equal amount of lysate protein electrophoresed on either 10% or 12.5% gel in the presence of SDS. The protein bands on the gel were transferred to membranes and the membrane immunoblotted with the respective antibodies described in the Results and discussion section for different experiments. The protein bands on the membrane were visualized by ECF and Phosphorimaging. The membranes were reprobed for the protein loading control actin or unphosphorylated proteins.

Measurement of caspase-3 and -9 activities in 1-LN prostate cancer cells with anti-CTD antibody. 1-LN prostate cancer cells in as above were treated overnight with: (1) vehicle; (2) anti-CTD antibody (1:200); (3) PFT-α (25 μM); (4) PFT-α (25 μM) then anti-CTD (1:200) antibody; or (5) staurosporine (5 μM) and incubated at 37 °C in a humidified CO2 (5%) incubator. Reactions were terminated by aspirating the medium, a volume of ice-cold PBS added, cells scraped into separate new tubes, and washed with PBS by centrifugation at 10,000g at 40 °C for 3 min. The respective pellets were lysed in a volume of lysis buffer B (50 mM HEPES, pH 7.4, 0.1% CHAPS, 0.1 mM EDTA and 5 mM DTT) and protein contents determined. Caspase-3 and -9 activities were assayed using the fluorogenic substrates AcDEVD-AMC for caspase-3 and AcLEHD-AMC for caspase-9 in 70 μl of reaction buffer containing 50 mM HEPES pH 7.4, 100 mM NaCl. 0.1% CHAPS, 10 mM DTT, 1 mM DTT, 1 μM EDTA and 10% glycerol, 20 μl of lysate (20–25 μg protein) and 10 μl of the respective substrates (300 μM) in 96-well plates. The reactions were carried out for 3 h at 37 °C. Caspase activities were determined by quantifying the levels of free AMC released by caspase hydrolysis of substrates in a fluorescent plate reader (excitation $\lambda = 360$ nm and emission $\lambda = 460$ nm) and expressed as relative fluorescent U/mg protein.

Statistical analyses of the data wherever appropriate were performed by student "*t*" test.

Results and discussion

PFT-α inhibits anti-CTD antibody-induced upregulation of p53 in 1-LN prostate cancer cells

A range of cellular stresses including DNA damage, oncogene activation, and hypoxia influence the levels of p53, its subcellular localization, post-translational modifications, and interaction with co-factors [19,20]. As a result of stabilization by these modifications, p53 suppresses oncogenic potential by mediating DNA repair, cell cycle arrest, or triggering apoptotic cell death. Elicitation of these effects by p53 is either transcription-dependent or independent [19,20]. In an earlier report we showed that the treatment of 1-LN prostate cancer cells with anti-CTD antibody significantly inhibited DNA synthesis, cell proliferation, and induced apoptosis [9]. This antibody also elevated the levels of p53, phosphorylated p53, and acetylated p53 in prostate cancer and melanoma cells. To further characterize the mediation of p53 in anti-CTD antibody-induced apoptosis, we treated cells with PTF-α and compared levels of p53 with cells treated with the p53 upregulator doxorubicin or anti-CTD antibody (Fig. 1A). Like doxoribucin, anti-CTD antibody caused a two to three fold increase in p53 levels in 1-LN cells (Fig. 1A). Treatment of cells with doxorubicin and antibody together further elevated p53 levels (Fig. 1). In contrast, treatment of 1-LN cells with PFT-α did not cause any increase in p53 and phosphorylated p53 (Fig. 1B) and this is in accordance with the work of others [10,13,15,21]. Importantly, pretreatment of cells with PFT-α nearly abolished anti-CTD, induced elevation in p53 levels in 1-LN cells (Fig. 1).

PFT-α abrogates anti-CTD antibody-induced down regulation of p-MDM2 levels in prostate cancer cells

MDM2 is a primary regulator of p53 stability. MDM2 binds tightly to the NH_2-terminal transactivation domain of p53 and blocks expression of p53-regulated genes [19,22,23]. During DNA damage, the interaction between p53 and MDM2 is reduced which allows p53 to accumulate. Post-translational modification of MDM2 and p53 regulate their interaction. Phosphorylation of MDM2 by Akt at Ser^{166} and Ser^{186} activates MDM2 and down regulates p53 [19,22,23]. Treatment of 1-LN prostate cancer cells with PFT-α caused a several fold enhancement in p-MDM2 levels (Fig. 1B). These upregulated p-MDM2 levels would promote its interaction with p53 causing its ubiquitination and degradation. These results further demonstrate that anti-CTD-antibody-induced apoptosis in prostate cancer cells is mediated by p53.

Anti-CTD antibody-induced down regulation of ERK1/2 activation is reversed by PFT-α in prostate cancer cells

The Ras-dependent MAPK pathway is one of the major signaling pathway in promoting mitogenesis and cellular proliferation in 1-

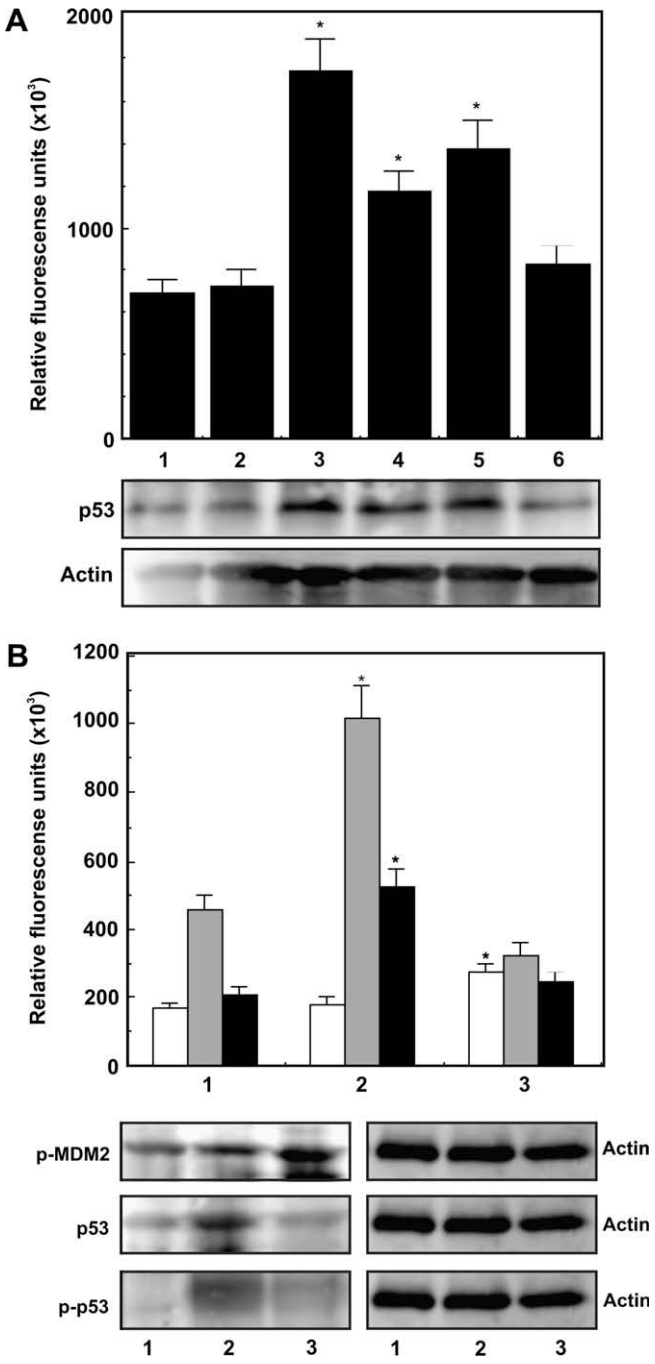

Fig. 1. Modulation of p53 activation. (A) A representative immunoblot of three experiments of p53 in cells treated with: (1) buffer; (2) anti-CTD antibody (1:200/30 min); (3) doxorubicin (5 μM/1 h); (4) doxorubicin then anti-CTD antibody; (5) PFT-α (25 μM/3 h) and (6) PFT then anti-CTD antibody. Changes in the protein levels of p53 due to these treatments are shown in the bar diagram above the immunoblot in arbitrary units ($\times 10^3$) expressed as mean ± SE from three experiments. Values in columns 3, 4, and 5 are significantly different from columns 1, 2, and 6 at the 5% levels are marked as ∗. (B) A representative immunoblot of three to four experiments showing changes in p53 (▨); p-p53 (■) and p-MDM2 (□) in 1-LN cells treated with: (1) buffer; (2) anti-CTD antibody (1:200/30 min) and (3) PFT-α (25 μM/3 h). Changes in levels of p-MDM2, p53, and p-p53 as a result of these treatments are shown in the bar diagram above the immunoblot as the ratio of test protein/actin expressed as mean ± SE from three experiments. Values in column 2 are significantly different at the 5% level from columns 1 and 3 as indicated by ∗. Protein loading controls in (A) and (B) are shown below the respective immunoblots.

LN cells stimulated with α_2M^* [15]. In response to diverse stimuli, MAPKs are activated through reversible phosphorylation of both Tyr and Thr residues by upstream kinases. They often phosphorylate a number of substrates and activate many signaling pathways which lead to diverse outcome including proliferation or apoptosis [24,25]. Treatment of prostate cancer cells with anti-CTD apoptosis upregulates p53 signaling and induces apoptosis [9]. In the preceding sections, we showed that treatment of cancer cells with anti-CTD antibody or doxorubicin-induced increase in p53 signaling, which was suppressed by PFT-α. In view of the greatly reduced receptor activation and cell surface expression of GRP78 observed in 1-LN cells incubated with anti-CTD antibody [9], one would anticipate down regulation of ERK1/2 activation. Incubation of 1-LN cells with anti-CTD antibody or doxorubicin inhibited α_2M^*-induced activation of ERK1/2. PFT-α treatment alone caused Erk1/2 activation which was suppressed by anti-CTD antibody or doxorubicin (Fig. 2). Activation of ERK1/2 by PFT-α has also been reported by others [15].

Anti-CTD antibody-induced upregulated expression of pro-apoptotic proteins and caspase activation is reversed by pretreatment with PFT-α in 1-LN cells

The mitochondrial "intrinsic" pathway and the death receptor "extrinsic" pathway are the two principal pathways leading to apoptosis. Both these pathways converge on caspase activation. Both pathways share effector caspases (-3, -6, and -7) which cleave cellular substrates leading to apoptotic cell death. Caspase-9 and -8 are initiator caspases for intrinsic and extrinsic pathways, respectively. The intrinsic pathway, also called the Bcl-2 regulated or the mitochondrial pathway, includes BAX, and BAK which when activated oligomerize and insert into the mitochondrial outer membrane to release cytochrome C which induces the formation of apoptosomes consisting of cytochrome C, Apaf-1, and procaspase-9. p53 initiates apoptosis both via the intrinsic and extrinsic pathways [26,27]. p53 increases the expression of cellular death

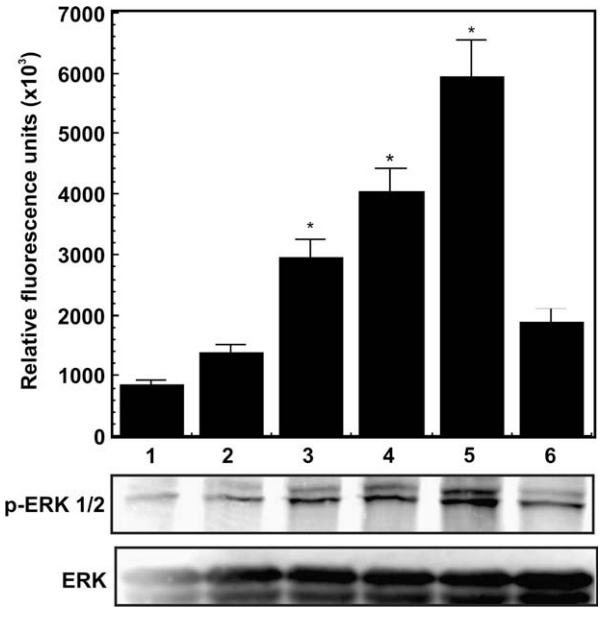

Fig. 2. Modulation of anti-CTD antibody and PFT-α-induced changes in the activation of ERK1/2 in 1-LN cells. A representative immunoblot of three experiments showing protein levels of p-ERK1/2 on treatment with: buffer (1); anti-CTD antibody (1:200/30 min) (2); PFT-α (25 μM/3 h) (3); PFT-α then anti-CTD antibody (4); α_2M^* (50 pM/20 min) (5), and doxorubicin (5 μM/1 h) (6). Changes in p-ERK1/2 is shown in arbitrary units ($\times 10^3$) and expressed as mean ± SE from three experiments. The immunoblotted protein loading control ERK1/2 is shown below the test immunoblot. Values in columns 3, 4, and 5 are significantly different from columns 1, 2, and 6 at the 5% level as indicated by ∗.

receptors and transcriptionally upregulates the expression of apoptosis-associated genes. BH_3-only proteins BAD, BID, BIK, NOXA, and PUMA function by physical interactions with the other Bcl-2 family members either resulting in inhibition of the antiapoptotic members or activation of the pro-apoptotic multidomain members [26,27]. PUMA and NOXA are both targets and mediators of p53-induced apoptosis and are induced in response to DNA damage. PUMA binds to and neutralizes all prosurvival members of the Bcl-2 family to promote apoptosis [26,27]. Pretreatment of cancer cells with PFT-α nearly abolished anti-CTD antibody-induced increase in PUMA, NOXA, BAD, BAX, and BAK. By contrast, PFT-α increased prosurvival Bcl-2 levels (Fig. 3A and B). Likewise,

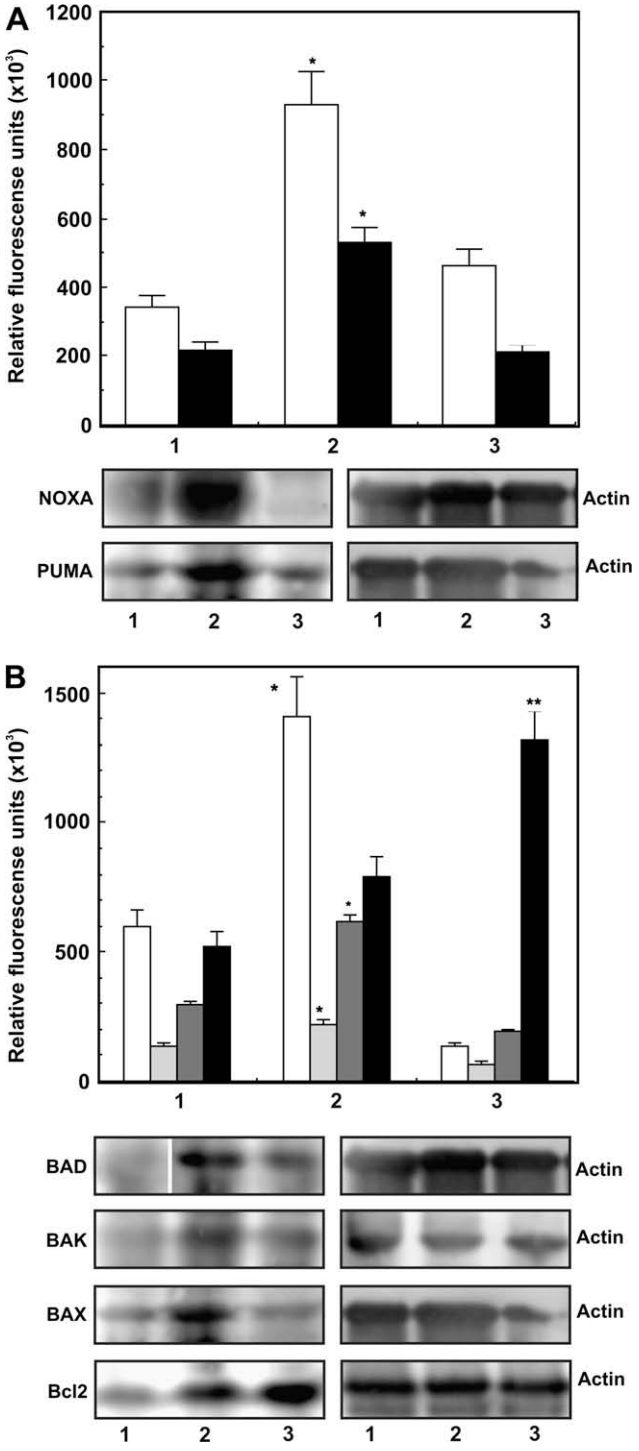

Fig. 3. Pretreatment with the p53 inhibitor PFT1-α reverses anti-CTD antibody-induced upregulation. (A) NOXA and PUMA (B), BAD, BAX, and BAK; and restoration of Bcl-2 levels (B). Changes in proteins in 1-LN cells treated with: buffer (1); anti-CTD antibody (1:200/30 min) (2); and PTF-α (25 μM/3 h) then anti-CTD antibody (1:200/30 min) (3) are expressed as ratios ± SE of these proteins/actin loading control. The bars in (A) are: NOXA (■) and PUMA (□). In (B) the bars are: BAD (□), BAK (▩), BAX (▩), and Bcl-2 (■). Each immunoblot was reprobed for actin and a representative immunoblot of actin for each of the respective proteins is shown. Values in column 2 (A and B) are significantly different from columns 1 and 3 (A and B) at the 5% level as indicated by ∗. Values in column 3 (B) are significantly different from columns 1 and 2 at the 5% level as indicated by ∗∗.

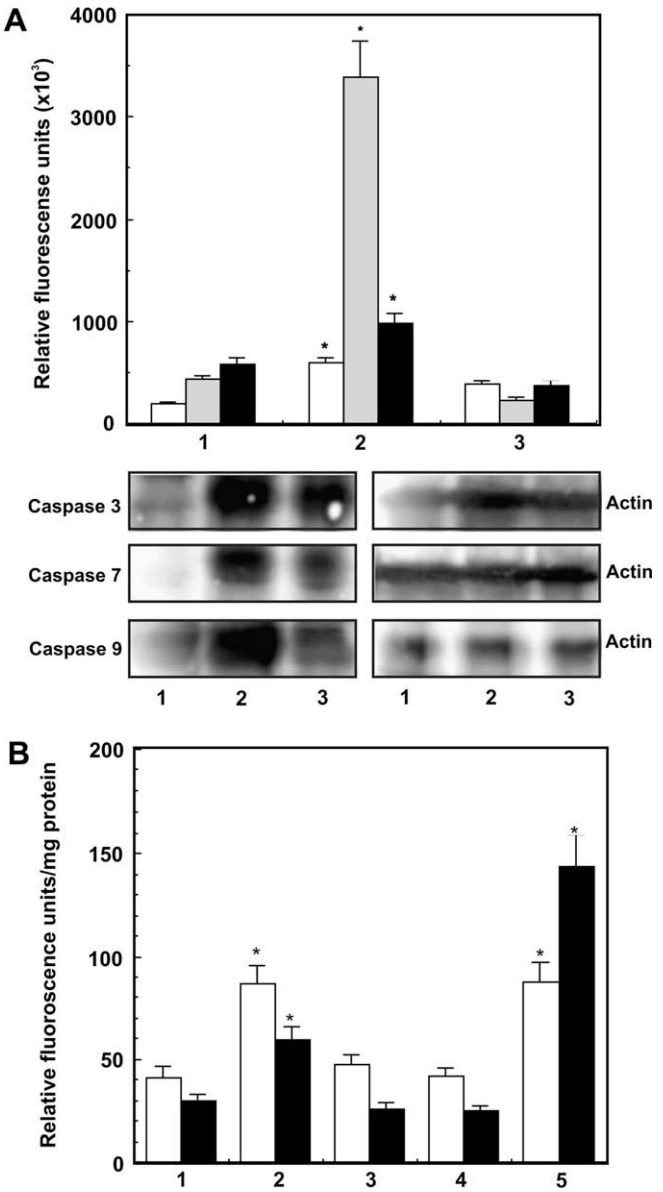

Fig. 4. Inhibition of anti-GRP78 antibody-induced activation of caspases-3 and -9 by pretreatment with PFT1-α in 1-LN prostate cancer cells. The bars in (A) are: activated caspase-3 (■), caspase-7 (▩), and caspase-9 (□). The lanes are: (1) buffer; (2) anti-GRP78 antibody A (1:200/30 min) and (3) PFT1-α (25 μM/3 h) then anti-CTD antibody. Representative immunoblots from three individual experiments are shown below the respective panels. Each immunoblot was reprobed for actin and a representative actin immunoblot for each of the respective proteins is shown. *Values in column 2 (A) are significantly different from columns 1 and 3 at the 5% level. (B) See Materials and methods for details. The bars are: activated caspase-3 (□) and caspase-9 (■). The lanes are: buffer (1); anti-CTD antibody (1:200/16 h) (2); PFT1-α (25 μM/16 h) (3); PFT1-α (25 μM/16 h then anti-CTD antibody) (4); and staurosporine (5 μM/16 h) (5). The caspase activities are expressed in arbitrary units as relative fluorescence/mg protein and are the mean ± SE from two experiments performed in duplicate. Values in columns 2 and 5 are significantly different from columns 1, 3, and 4 at the 5% level as indicated by ∗.

pretreatment of cells with PFT-α prevented anti-CTD antibody-induced activation of caspase-3, -7, and -9 (Fig. 4A and B).

Conclusion

We report here that anti-CTD antibody-induced apoptosis in prostate cancer cells is mediated by upregulation of p53 which activates mitochondrial apoptotic signaling resulting in caspases activation, and PFT-α pretreatment of cells blocks anti-CTD antibody-induced increase in p53, pro-apoptotic protein levels, and caspase activation.

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