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p202, an interferon-inducible protein, inhibits E2F1-mediated apoptosis in prostate cancer cells

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Abstract

p202, an interferon (IFN) inducible protein, is a phosphonuclear protein involved in the regulation of cell cycle, apoptosis, and differentiation. E2F1 belongs to the E2F family of proteins that are important cell cycle regulators in promoting cell growth. On the other hand, the deregulated expression of E2F1 also triggers apoptosis independent of p53 status. It has been well documented that p202 is able to inhibit cell growth by binding to E2F1 and abolishing the E2F1-mediated transcriptional activation of S-phase genes. However, it is not known whether E2F1-mediated apoptosis can be counteracted by p202 expression. Here, we show that E2F1-mediated apoptosis induced by the infection of an E2F1-expressing adenoviral vector (Ad-E2F1) was greatly diminished in p202-expressing prostate cancer cells. The E2F1-mediated caspase-3 activation was also reduced in p202-expressing cells infected with Ad-E2F1. Since caspase-3 is one of the E2F1 transcriptional targets, this result is consistent with the ability of p202 to inhibit the transcriptional activity of E2F1. Therefore, our results suggest a possible link between the IFN and E2F pathways in regulating apoptosis.

p202, a mouse interferon (IFN)-inducible protein, belongs to HIN-200 (hematopoietic interferon-inducible nuclear proteins with 200-amino-acid repeat) protein family in which the family members share a common structural feature of one or two 200 amino acid long homologous motifs [1–3]. The functional significance of p202 in cellular regulation is implicated by its physical interaction with several transcriptional factors that regulate cell cycle, including E2F1 [4]. These protein–protein interactions generally lead to transcriptional repression of promoters that are activated by these transcriptional factors.

E2F1 is a member of the E2F transcription factor family. Although E2F1 plays an important role during S-phase entry, it has been shown that overexpression of E2F1 is associated with induction of apoptosis [5–8]. p202 binds to the DNA binding domain of E2F1 and thus prevents E2F1 from binding to DNA. Consequently, this protein–protein interaction leads to transcriptional inhibition of many S-phase genes activated by E2F1, resulting in attenuation of S-phase entry [9]. However, it is not known if p202 can affect E2F1-mediated apoptosis. In this report, we demonstrate that while the infection of a recombinant adenoviral vector that expresses E2F1 (Ad-E2F1) readily induced apoptosis in a prostate cancer cell line, PC3, this E2F1-mediated apoptosis is significantly diminished in p202-expressing PC3 cells. Consistent with the above observation, we show that Ad-E2F1 infection correlates with the activation of an apoptotic molecule, caspase-3, that is inactivated in p202-expressing cells.

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Materials and methods

Cell lines and adenovirus vectors. The human prostate cancer cell line, PC-3 [10], was cultured in DMEM/F12 media supplemented with 10% fetal bovine serum. The plasmids, pcDNA3 (Invitrogen, San Diego; CA) and CMV-p202 [9], were transfected into PC-3 cells. Four p202-expressing PC-3 stable clones (p202-1, -2, -3, and -4) were isolated [11]. The G418-resistant colonies from pcDNA3 transfection were pooled as a control. The construction of Ad-E2F1 has been described previously [6].

Western blot and apoptosis assays. Western blot analysis and PARP analysis were performed as described previously [6,12]. For flow cytometry analysis, cell lines were mock infected, or treated with Ad-Luc or Ad-E2F1 at an MOI of 100. Forty-eight hours following infection, cells were collected and then washed with PBS. Cell pellets were then fixed in 80% ethanol and kept a 4 °C until immediately prior to flow cytometric analysis. Cell pellets were then centrifuged, washed with PBS, and resuspended in propidium iodide solution (0.1% sodium citrate, 0.1% Triton X-100, 20 μg RNAse/ml, and 50 μg propidium iodide). Cells were then analyzed for DNA content (Epics Profile, Coulte Miami, Fla.).

Caspase-3 fluorometric assay. A caspase-3 fluorometric assay kit was utilized (R&D systems, Minneapolis, MN). Briefly, the Ad-E2F1, Ad-Luc (recombinant adenovirus expressing the luciferase reporter gene), and mock-infected cells were harvested at 48 h post-infection. Cell pellets were lysed with 25 μl/1×10^6 cells of cold lysis buffer (provided by the manufacturer). Fifty μl of cell lysate was then mixed with 50 μl of 2× reaction buffer (provided by the manufacturer) and 5 μl caspase-3 fluorogenic substrate (DEVD-AFC) in a 96-well plate. The reaction was incubated at 37 °C for 1 – 2 h followed by fluorescence analysis using a fluorescent microplate reader with light excitation at 400 nm and light emission at 505 nm.

Results and discussion

To test whether p202 affects the apoptosis induced by E2F1, we utilized Ad-E2F1 (MOI of 100) [6] or control adenovirus expressing the luciferase reporter gene (Ad-Luc) to infect four p202-expressing PC-3 stable cell lines (p202-1, -2, -3, and -4) [11] as well as the parental PC-3 and the pooled empty-vector (pcDNA3) transfected PC-3 cells. To confirm the expression of E2F1 in Ad-E2F1-infected cells, we performed a Western blot to detect E2F1 expression using an E2F1-specific antibody. As shown in Fig. 1, E2F1 is readily detectable in Ad-E2F1-infected cells (E) but not in the mock (C)- or Ad-Luc (L)-infected cells. To examine the effect of p202 expression on E2F1-mediated apoptosis, we subjected the virus (Ad-E2F1 or Ad-Luc)-infected cells to flow cytometry analysis in order to assess the percentage of sub-G1 (apoptotic) cell population. The percentage of apoptosis in the Ad-E2F1-infected cells was normalized to the Ad-Luc-treated cells, i.e., percentage of sub-G1 of Ad-E2F1 infection/percentage of sub-G1 of Ad-Luc infection (Fig. 2A). While it is expected that Ad-E2F1 induced apoptosis in PC-3 and pcDNA3 vector-transfected control cells, the E2F1-induced apoptosis is greatly attenuated in all p202-expressing cells. To further confirm this observation, we employed another apoptosis assay, i.e., poly(ADP-ribose) polymerase (PARP) cleavage assay, in which intact 116 kDa PARP is cleaved by caspases into a fragment of approximately 85 kDa [12]. The cell lysate isolated from Ad-Luc (L) and Ad-E2F1 (E) treated cells was analyzed by Western blot using PARP-specific antibody. As shown in Fig. 2B, while Ad-E2F1 infection resulted in PARP cleavage indicative of apoptosis in PC-3 and pcDNA3 vector-transfected cells, the E2F1-induced PARP cleavage was significantly inhibited in p202-2 (a p202-expressing cell line that has relatively higher p202 expression than the other p202 stable cell lines [11]). Taken together, these results strongly indicate that p202 is able to inhibit apoptosis induced by overexpression of E2F1. Since a variety of substrates including PARP [13] are cleaved during the cascade of caspase activation leading to apoptosis, our results suggest that the activation of caspases may be involved in Ad-E2F1-induced apoptosis. Furthermore, recent report suggests that the activation of caspase-3 is, at least in part, responsible for the E2F1-mediated apoptosis [14]. To test whether the inhibition of E2F1-mediated apoptosis in p202-expressing cells correlates with the inhibition of caspase-3 activity, we performed the caspase-3 fluorometric assay. Briefly, the cell lysates isolated from Ad-E2F1 and Ad-Luc infected cells as well as the untreated cells were incubated with caspase-3-specific fluorogenic substrate (DEVD-AFC) followed by fluorescence analysis at 505 nm. The intensity of fluorescence is indicative of caspase-3 activity. As shown in Fig. 3, Ad-E2F1 infected PC-3 and pcDNA3 vector-transfected cells exhibited higher caspase-3

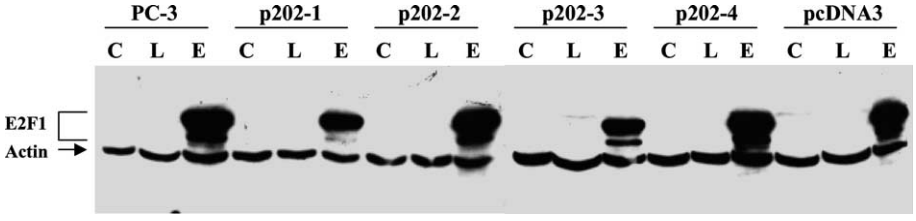

Fig. 1. E2F1 overexpression by Ad-E2F1 in human prostate cancer cells. Parental PC3 cells, pcDNA3 vector-transfected cells, and p202-expressing cells (p202-1, p202-2, p202-3, and p202-4) were mock infected (C), or infected with Ad-Luc (L) or Ad-E2F1 (E) at an MOI of 100. Forty-eight hours following infection, cell lysates were prepared and 50 μg of protein was subjected to Western blot analysis. Nylon membranes were probed with antibodies to E2F1 and actin. Actin expression was used to confirm equal protein loading.

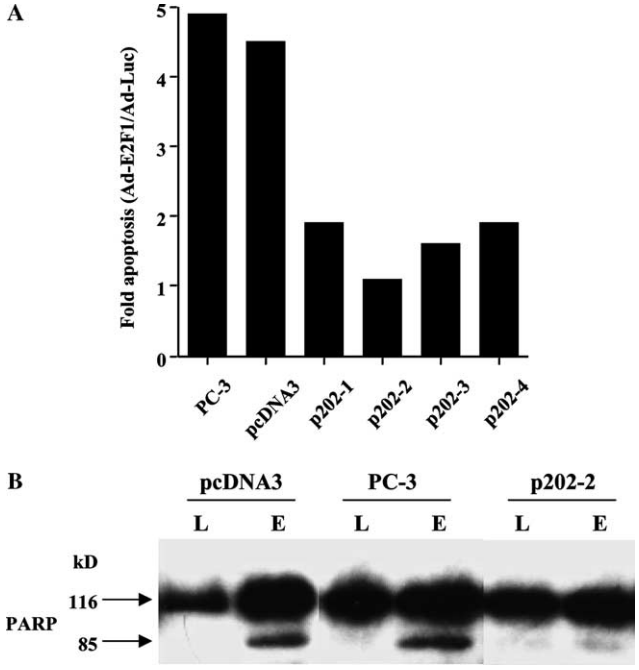

Fig. 2. Attenuation of E2F1-mediated apoptosis in p202-expressing prostate cancer cells. (A) Apoptosis determined by flow cytometry assay. Cell lines were mock infected, or treated with Ad-Luc or Ad-E2F1 at an MOI of 100. Forty-eight hours following infection, cells were harvested and subsequently analyzed by flow cytometry (Materials and methods). The subdiploid population was calculated and recorded as percentage of apoptotic cells. (B) PARP cleavage was assessed by Western blot analysis. Protein was collected from cells following treatment with Ad-E2F1 (E) or Ad-Luc (L). Protein extracts were subjected to gel electrophoresis and then transferred to a nylon membrane. Membranes were probed with an antibody against PARP. The arrows indicate the full-length PARP (116 kDa) and the cleaved fragment (85 kDa).

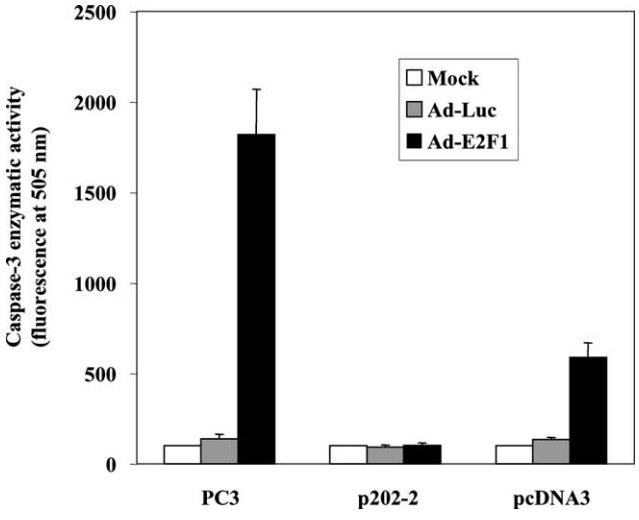

Fig. 3. The level of caspase-3 activity is measured by caspase-3 fluorometric assay. The cells were infected by Ad-Luc or Ad-E2F1 for 48 h. Untreated cells were used as control. The cell lysates were then tested for caspase-3 activity by the addition of a caspase-specific peptide that is conjugated to the fluorescent reporter AFC. The fluorescence signals were detected at 505 nm after being excited by light at 400 nm wavelength.

activity than the controls (mock infection or Ad-Luc infection). In contrast, the caspase-3 activity of the Ad-E2F1-infected cells expressing p202 was at the basal level of those infected with Ad-Luc or without treatment. This result suggests that caspase-3 inactivation is involved in the inhibition of E2F1-mediated apoptosis by p202.

It has been shown that p202-mediated growth retardation is associated with the inactivation of E2F1 via p202/E2F1 interaction [9]. In this report, we show that p202 is able to inhibit E2F1-mediated apoptosis and that this is associated with the inactivation of caspase-3. Given the observation that E2F1 induces caspase-3 expression, it is likely that p202 inhibits the E2F1-mediated apoptosis by abolishing the transcriptional activity of E2F1 and that in turn represses caspase-3 expression, leading to attenuation of apoptosis. Several lines of evidence seem to suggest that the interacting protein partners determine the functional role of p202 in apoptosis. For instance, p202 binds c-Myc and inhibits c-Myc-mediated apoptosis [15]. p202 could also attenuate the apoptosis induced by p53, presumably by inactivating the p53 transcriptional activity via p202/p53 interaction (K. Hunt, unpublished results, [16,17]). In contrast, we have shown that p202 interacts with the anti-apoptotic molecule, NF-κB, and sensitizes cells to apoptosis induced by TNF-α [12,18]. Together, these observations demonstrate the versatility of p202 in controlling the cell fate by interacting with different cell cycle regulators or survival factors in response to different extracellular stimuli such as growth factor or cytokines. Therefore, our results presented here suggest a possible cross talk between the IFN and E2F pathways in regulating apoptosis.

Acknowledgments

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