

Induction of colonic epithelial cell apoptosis by p47-dependent oxidants¹

Ying Gu, Rhonda F. Souza, Ru Feng Wu, You Cheng Xu, Lance S. Terada*

Department of Internal Medicine, University of Texas Southwestern and the Dallas VAMC, Mail Code 151, 4500 S. Lancaster Rd., Dallas, TX 75216, USA

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Abstract Exogenous oxidants appear capable of initiating both proliferative and death signals, but the role of endogenous oxidants in either tumorigenesis or tumor suppression is unclear. We found that expression of the NAD(P)H oxidase adapter p47^{phox} was suppressed in human colon carcinoma specimens relative to adjacent normal colon. Overexpression of p47^{phox} increased apoptosis in colon cancer cell lines independent of p53 and mismatch-repair competency. p47^{phox} was found to interact with the c-Abl adapter Abl interactor-1 (ABI-1), and p47^{phox} coprecipitated with both ABI-1 and c-Abl. Ectopic expression of p47^{phox} in colon cancer cells increased oxidant production with phosphorylation and activation of nuclear c-Abl and consequent apoptosis. Colonic epithelial p47^{phox} may be specifically targeted to a c-Abl-containing complex that serves a physiologic tumor suppressing function.

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Key words: Oxidants; Superoxide; NADPH oxidase; Colon cancer; c-Abl; Abl interactor-1

1. Introduction

Reactive oxidant species have long stood accused of promoting tumor formation, largely through mutagenic events which accompany direct DNA base and sugar modification and strand breaks [1]. Indeed, oxidant-induced apoptosis has generally been linked to induction of p53 as a response to such broad DNA damage [2,3]. More recently, endogenously produced oxidants have been recognized as mitogenic signaling agents, providing an additional pathway by which reactive oxidants may facilitate cell transformation. Consistently, an NAD(P)H oxidase has been implicated as the source of these mitogenic oxidants. The proliferative effects of angiotensin II, for instance, can be blocked by treatment with antisense oligonucleotides for p22^{phox} [4], and overexpression of the phagocyte oxidase paralog Nox1 (Mox1) transforms cells [5].

Although much is known about the molecular events surrounding colon carcinogenesis, a specific role for oxidants has not been identified. Normal human colon appears to express significant levels of the NAD(P)H oxidase cytochrome b₅₅₈, consisting of p22^{phox} and gp91^{phox} [6], suggesting a potential role for this oxidase in colon tumorigenesis. In both phago-

cytic and non-phagocytic cells, physiologic activation of the NAD(P)H oxidase appears to require the signal-receiving adapter p47^{phox} [7,8]. In the present study, we found that normal colon indeed expressed p47^{phox}, but surprisingly, expression of p47^{phox} in colon carcinoma specimens was decreased relative to adjacent normal colon tissue. Overexpression of p47^{phox} by colon carcinoma cell lines increased apoptosis rates, suggesting a tumor suppressing rather than promoting role of endogenous oxidants. p47^{phox}-derived oxidants appear to activate nuclear c-Abl in a p53-independent mechanism of cell death.

2. Materials and methods

2.1. Quantitative RT-PCR

Total RNA was extracted from eight unselected colon carcinoma resection specimens and adjacent normal colon using Trizol (Gibco). A truncated p47^{phox} cDNA mimic was created by removal of a 180 base Bg/II fragment, and p47^{phox} expression was assessed using an RT-linked competitive PCR assay [8].

2.2. Plasmid and adenoviral construction

All PCR amplifications for subcloning or mutagenesis were performed with Pfu Turbo (Stratagene). Ad-p47 and Ad-lacZ were constructed previously [9]. Ad-p47(W193R) was constructed using the AdenoX system (Clontech). The yeast shuttle vectors pGBKT7-p47 and pGBKT7-p47(1–298) were previously constructed [9]. Truncations of p47^{phox} were PCR amplified between *Eco*RI and *Sal*I sites with appropriate stop codons inserted and ligated into pGBKT7. p47(S303,304,328D) was derived by sequential PCR mutagenesis of wild type p47^{phox}, followed by subcloning into pGBKT7. pGEX-p47 and pGEX-p47(1–298) were derived by amplification of p47^{phox} between *Eco*RI sites and ligation into pGEX-2TK. Full-length Abl interactor-1 (ABI-1) was recovered from a library clone and subcloned into the *Eco*RI and *Sal*I sites of pCIneo-FLAG, with frame correction. All mutations and constructs were confirmed by sequencing.

2.3. Apoptosis assays and oxidant production

Rapidly growing Caco-2 and SW48 cells were trypsinized and inoculated with adenoviruses (MOI = 100:1) immediately after replating. Transgene expression was 50–70% as assessed by *lacZ* expression. After 24 h, media was replaced and after another 24 h, apoptosis was assessed. STI571 (gift of Novartis Pharma) was used at 10 μM for the last 24 h. Mono- and oligonucleosomal DNA fragments were detected with a cell death sandwich ELISA kit (Roche). Cell cycle analysis was determined 24 h after addition of adenoviruses by flow cytometry with propidium iodide [10]. Oxidant production was assessed with 2',7'-dichlorofluorescein-diacetate (10 μM) using flow cytometry [8].

2.4. Yeast two-hybrid screening and interaction assay

A human endothelial cell library was previously constructed [9]. *S. cerevisiae* strain AH109 (Clontech) was stably transformed with pGBKT7-p47(1–298) prior to large scale library transformation using lithium acetate. Colonies surviving auxotrophic selection for bait vector (tryptophan), prey vector (leucine), and interaction (both histidine and adenine) were tested for *lacZ* expression. Single clones from positive colonies were retested, and library plasmids from positives were

*Corresponding author. Fax: (1)-214-857 0340.

E-mail address: lance.terada@med.vu.gov (L.S. Terada).

¹ Nucleotide sequence data reported are available in GenBank database under accession # AF540955.

passed through *E. coli*, transformed into AH109 yeast, and mated to bait-containing Y187 yeast for confirmation of interaction. Interaction of ABI-1 with p47 truncations was tested by transformation of the p47 truncations into Y187, followed by mating with ABI-1-transformed AH109. Diploids were assessed for *lacZ* expression with a filter lift assay. ABI-1 was not an autonomous transactivator.

2.5. Glutathione S-transferase (GST) pulldown

GST, GST-p47, and GST-p47(1–298) were produced as previously described [9] and assessed by Coomassie. Full-length ABI-1 was in vitro translated from pAD-GAL4-ABI-1 with 15 μ Ci [35 S]methionine (TNT Quick Coupled, Promega), and 5 μ l of translation was added to each binding reaction. After extensive washing, products were analyzed by autoradiography.

2.6. Immunoprecipitation

Caco-2 or Fx-293 (Fx) cells infected with Ad-p47 or Ad-lacZ and/or transfected with pCINF-ABI-1 were lysed for 30 min at 4°C in 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4 , 1% Triton X-100, 1 mM β -glycerophosphate, 1 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF), then sonicated for 5 s and centrifuged at $19\,720\times g$ for 20 min. Following preclearing, complexes were immunoprecipitated with anti-c-Abl (Santa Cruz) or anti-p47 (Santa Cruz) and immunoblotted with anti-c-Abl, anti-p47 (rabbit polyclonal, kind gift of Dr. Bernard Babor), anti-FLAG (M2, Sigma), or anti-phosphotyrosine (Upstate).

2.7. c-Abl activity

c-Abl activity was assessed by autophosphorylation of immunoprecipitated c-Abl [11] in kinase buffer (10 mM Tris, pH 7.6, 5 mM EDTA, 130 mM NaCl, 1% Triton X-100, and protease inhibitors) containing 1 mM cold ATP and 10 μ Ci [γ - 32 P]ATP for 30 min at 30°C. Nuclear and cytoplasmic c-Abl were separated [12] following lysis in cold lysis buffer (1 mM EGTA, 1 mM EDTA, 10 mM KCl, 2 mM MgCl_2 , 10 mM β -glycerophosphate, 25 mM HEPES, 1 mM DTT, 0.05% Igepal CA630, 2 mM PMSF, 100 μ g/ml leupeptin, 100 μ g/ml aprotinin, and 0.5 mM Na_3VO_4) for 10 min, Dounce homogenization, and collection of nuclear fraction spun through a 1 M sucrose cushion at $1600\times g$ for 15 min. The cytoplasmic fraction was further clarified with a $150\,000\times g$ spin for 30 min.

3. Results and discussion

3.1. Overexpression of p47^{phox} increases colon carcinoma cell death

We found a consistent decrease in the expression of p47^{phox} in colon carcinoma specimens compared to adjacent normal colon tissue, with seven of eight carcinomas displaying reduced p47^{phox} transcript levels (Fig. 1). Adenoviral delivery of wild type p47^{phox} increased apoptotic death of both Caco-2 and SW48 colon carcinoma cell lines (Fig. 2a,b), suggesting a possible tumor-suppressive role for p47^{phox} in human colon tissue. In contrast, p47(W193R) had no effect on apoptosis. This latter mutant has been shown to lack binding function of the first p47 SH3 domain, a function critical to assembly of the oxidase [8,13]. Overexpression of p47^{phox} also increased oxidant production (Fig. 2c) and cell cycle exit of Caco-2 cells into an apoptotic sub-G₀ fraction (Fig. 2d). The antioxidant NAC prevented p47-induced cell death without affecting baseline rates of cell death, and the p47(W193R) mutant decreased the sub-G₀ fraction below baseline levels, also consistent with an oxidant-dependent pro-apoptotic effect of p47^{phox}.

The mechanism by which p47^{phox} increases oxidant production in Caco-2 cells is not clear, but may involve assembly of a traditional NADPH oxidase complex. RT-PCR of Caco-2 cell RNA between coding bases 1157–1636 demonstrated expression of the principal cytochrome *b*₅₅₈ subunit, gp91^{phox}, and sequencing of the PCR fragment revealed 100% nucleotide

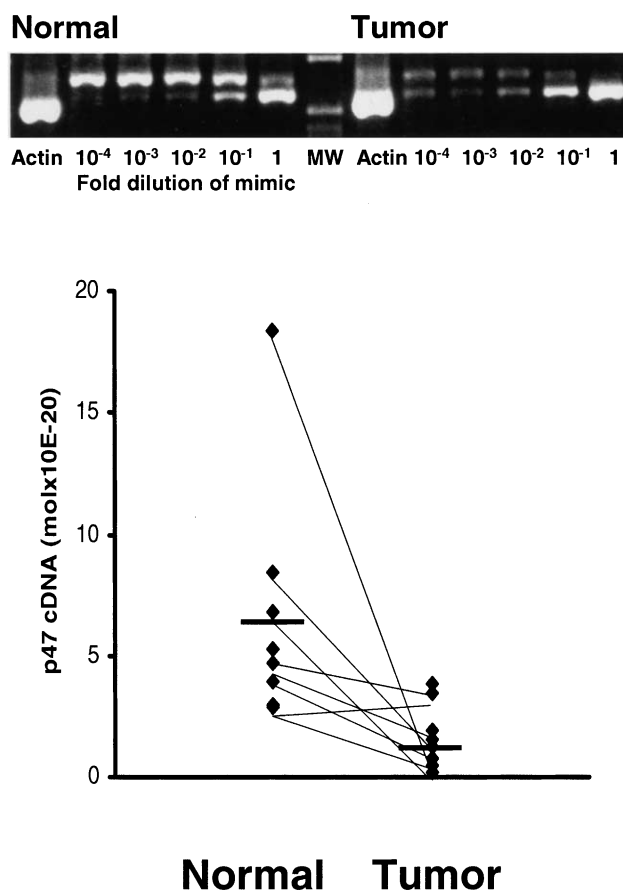


Fig. 1. p47^{phox} transcript levels are decreased in colon carcinoma. Total RNA from tumor and adjacent normal colon in resection specimens was subjected to quantitative RT-PCR analysis. Top panel shows representative competitive analysis, with authentic p47^{phox} band migrating slower than truncated mimic. p47^{phox} transcript levels per μ g total RNA were decreased in tumor specimens relative to adjacent normal colon ($P < 0.05$, paired *t*-test). Horizontal bars in lower panel represent group means.

identity. This finding is consistent with expression of the NADPH oxidase cytochrome by normal human colon [6]. Caco-2 cells have also recently been found to express the two other principle oxidase subunits, p22^{phox} and p67^{phox} [14]. Alternatively, Caco-2 cells have been shown to express the paralogous NOX1, which requires an adapter protein such as p47^{phox} for activation [15], although this latter cytochrome appears to transduce growth rather than death signals when ectopically expressed in Caco-2 cells [5].

3.2. p47^{phox} associates with ABI-1

To identify a potential mechanism for the induction of apoptosis by p47^{phox}, we screened a human library for p47-interacting partners using a truncated p47^{phox} [p47(1–298)] lacking the autoinhibitory C-terminus. Truncation of this region appears to open domains of p47^{phox} necessary for binding function [16]. We retrieved a cDNA encoding the c-Abl adapter ABI-1 (e3B1/SSH3BP1), and confirmed the interaction in yeast mating experiments (not shown). The cDNA includes the full coding region of ABI-1 and reveals a presumed splice variant (GenBank accession # AF540955). Compared with the longest reported transcript (GenBank # NM005470), the predicted protein lacks amino acid residues 155–159 and 274–

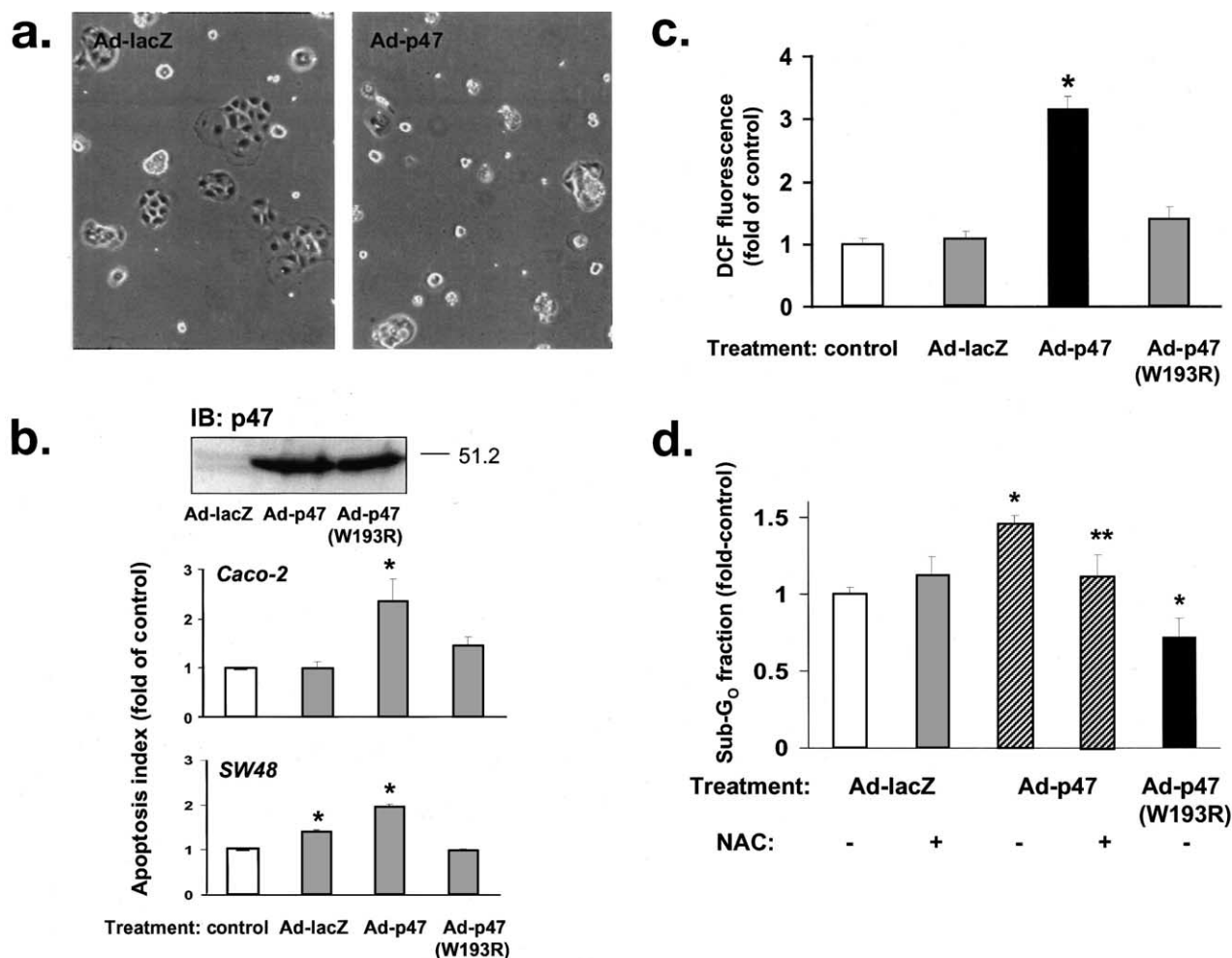


Fig. 2. p47^{phox} overexpression increases colon cancer cell apoptosis. a: Caco-2 cells were infected (MOI = 1:100) with either Ad-lacZ or Ad-p47 for 24 h, and examined with phase contrast microscopy (4×). Compared with the Ad-lacZ-infected cells (left panel), Ad-p47-infected cells demonstrated a number of condensed, highly refractile cells. b: Top panel shows immunoblot with anti-p47 of Caco-2 cells infected with Ad-lacZ, Ad-p47, or Ad-p47(W193R). DNA fragmentation, assessed by sandwich ELISA, increased in Caco-2 and SW48 cells 24 h after infection with Ad-p47 (**P* < 0.05 compared to control). Infection with Ad-p47(W193R) had no effect compared to control, but decreased DNA fragmentation in SW48 cells compared to Ad-lacZ-infection (*P* < 0.05). c: Oxidant production, measured as dichlorofluorescein (DCF) fluorescence, was assessed in Caco-2 cells after 24 h exposure to respective adenoviruses. Oxidant production increased after infection with Ad-p47 but not Ad-lacZ or Ad-p47(W193R) (**P* < 0.05 compared to control). d: Cell death was also assessed in Caco-2 cells after 24 h infection with respective viruses by the appearance of cells in the sub-G₀ cell cycle fraction. Infection with Ad-p47 increased exit of cells into the sub-G₀ fraction (**P* < 0.05 compared to Ad-lacZ). Treatment of Ad-p47-infected cells with NAC (5 mM, pH adjusted, present for the final 16 h) decreased the sub-G₀ fraction back to baseline levels (***P* < 0.05 compared to Ad-p47 without NAC), and infection with p47(W193R) decreased this value below baseline (**P* < 0.05 compared to lacZ).

300 and contains a single nucleotide polymorphism (T515C, encoding L172P). A direct interaction between p47(1–298) and, to a lesser extent, full-length p47^{phox}, was demonstrated using a GST pulldown assay (Fig. 3a). Yeast mating studies revealed an interaction between ABI-1 and the tandem SH3 domains of p47^{phox} [p47(153–286), Fig. 3b]. Specifically, a weak interaction between ABI-1 and the second p47 SH3 domain [p47(223–286)], and a robust interaction with the first p47 SH3 domain [p47(153–219)] was reproducibly demonstrated. Interestingly, ABI-1 also interacted with the first p47 SH3 region in which the tryptophan bridge of the SH3 binding surface was disrupted [p47(153–219;R193)], suggesting an alternate binding site contained within p47(153–219), and perhaps utilizing a mechanism independent of the classical SH3–polyproline helix interaction. As further evidence that the ABI-1 binding site was not an artifact caused by artificial p47 truncations, we demonstrated an interaction be-

tween ABI-1 and p47(S303,304,328D) (Fig. 3b). This latter full-length mutant mimics phosphorylation of three critical serines, and appears to induce unmasking of the tandem SH3 regions [16]. Finally, in Fx cells, FLAG-ABI-1 specifically coprecipitated with p47^{phox} (Fig. 3c).

ABI-1 is an ubiquitous protein, well-expressed in human colon [17], and is able to mediate both pro- and anti-proliferative signals. It associates with PDGF and EGF receptors via Eps8 [17], and transduces growth signals distal to Ras [18]. Conversely, overexpression of ABI-1 suppresses fibroblast growth [17], and overexpression of its mouse homolog Abi-1 inhibits v-Abl-induced transformation and ERK activation [19,20]. Not surprisingly, its binding partner c-Abl also acts as a molecular switch for cell fate, mediating both growth and death signals. Growth factors such as PDGF and EGF activate c-Abl [21], and the fusion protein BCR-ABL is a well known oncogene; however, overexpression of c-Abl induces

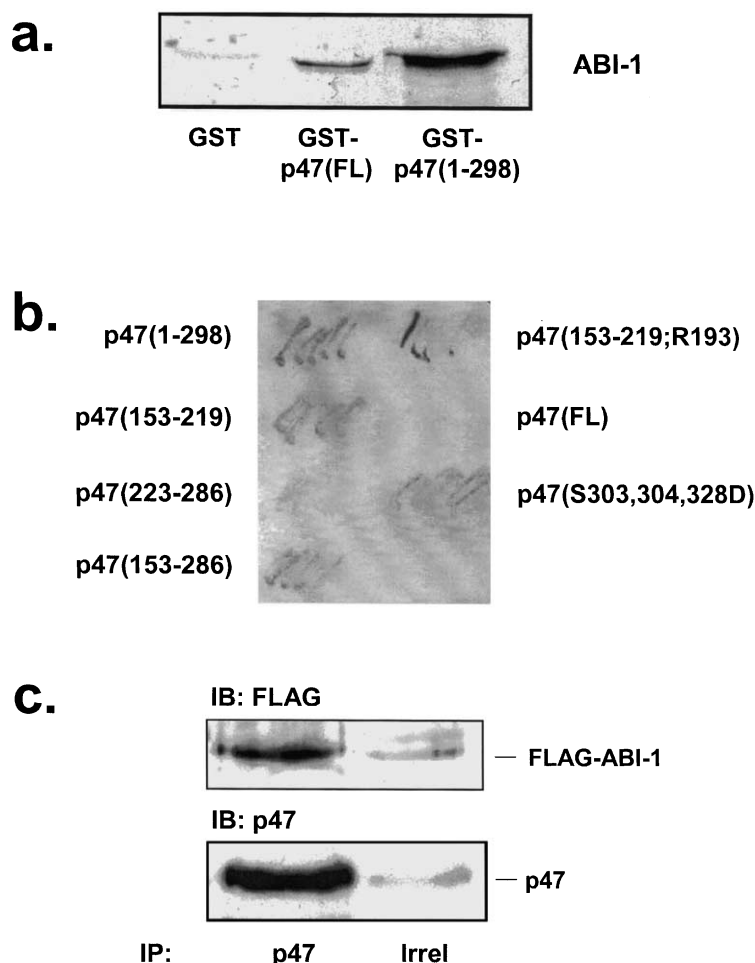


Fig. 3. ABI-1 binds $p47^{phox}$. a: Direct binding of ABI-1 to $p47^{phox}$ was assessed by a GST pull-down assay. [35 S]Methionine-labelled ABI-1 bound to GST fusions of both full-length $p47^{phox}$ and, more avidly, $p47(1-298)$, but not to GST. b: AH109 yeast transformed with GAL4-AD-ABI-1 were mated with Y187 yeast transformed with GAL4-BD- $p47$ truncations, as indicated. Diploids were replated and lacZ expression of lifted colonies is shown. ABI-1 interacted primarily with a region coinciding with the first SH3 domain [$p47(153-219)$], despite disruption of the SH3 surface [$p47(152-219;R193)$]. ABI-1 also interacted with an unfolded form of full-length $p47^{phox}$ [$p47(S303,304,328D)$], and weakly with a region corresponding to the second SH3 domain [$p47(223-286)$]. Representative of three experiments. c: Fx cells were transfected with pCINF-ABI-1 and 24 h later infected with Ad- $p47$. After a subsequent 24 h, $p47$ was immunoprecipitated and blots were probed with anti-FLAG for FLAG-ABI-1 (top), then anti- $p47$ for $p47^{phox}$ (bottom).

apoptosis [22], and endogenous c-Abl is required for apoptosis precipitated by DNA damage [23]. Neither ABI-1 nor c-Abl is presently thought to participate in colon tumorigenesis or tumor suppression.

3.3. $p47^{phox}$ overexpression increases activation of c-Abl

Because high levels of exogenous oxidants can activate and phosphorylate c-Abl [12,24], we next asked whether $p47^{phox}$ could mediate activation of c-Abl. We found first that endogenous c-Abl specifically coprecipitated with overexpressed $p47^{phox}$ (Fig. 4a), consistent with simultaneous binding of ABI-1 with $p47^{phox}$ and c-Abl. In addition, overexpression of $p47^{phox}$ increased tyrosine phosphorylation of endogenous c-Abl, whereas $p47(W193R)$, which binds ABI-1 (Fig. 3b) but does not support oxidant production (Fig. 2c), decreased c-Abl phosphorylation (Fig. 4b). Finally, overexpression of wild type $p47^{phox}$ increased whereas $p47(W193R)$ decreased c-Abl activity (Fig. 4c). The $p47$ -dependent increase in c-Abl activity was found predominantly in the nuclear fraction (Fig. 4d), consistent with the proposed cytostatic and apoptotic role

of nuclear as opposed to cytoplasmic c-Abl [25–27]. Indeed, the Abl kinase inhibitor STI571 decreased $p47$ -induced apoptosis, while having no effect on baseline apoptosis rates (Fig. 5). Recently, oxidants were shown to activate cytoplasmic, not nuclear, c-Abl to induce apoptotic death in COS7 cells [12]. In this latter study, however, cytoplasmic c-Abl was activated in response to a high dose (1 mM) of exogenous oxidants over 15 min, and the response in our experiments to a presumably lower level of site-directed, endogenous oxidants may differ. Alternatively, activated cytoplasmic c-Abl may translocate into the nucleus during certain conditions such as cell attachment [28], and our observations taken at a later stage may reflect a similar nuclear importation.

While apoptosis resulting from a bolus of exogenous oxidants appears to require the tumor suppressor p53 [2,3], we found that $p47^{phox}$ increased apoptosis in the p53-deficient Caco-2 cell line, again suggesting a different response to exogenous as opposed to endogenous oxidants. The lack of p53-dependence is consistent with involvement of c-Abl, since overexpression of c-Abl or activation of c-Abl by cisplatin

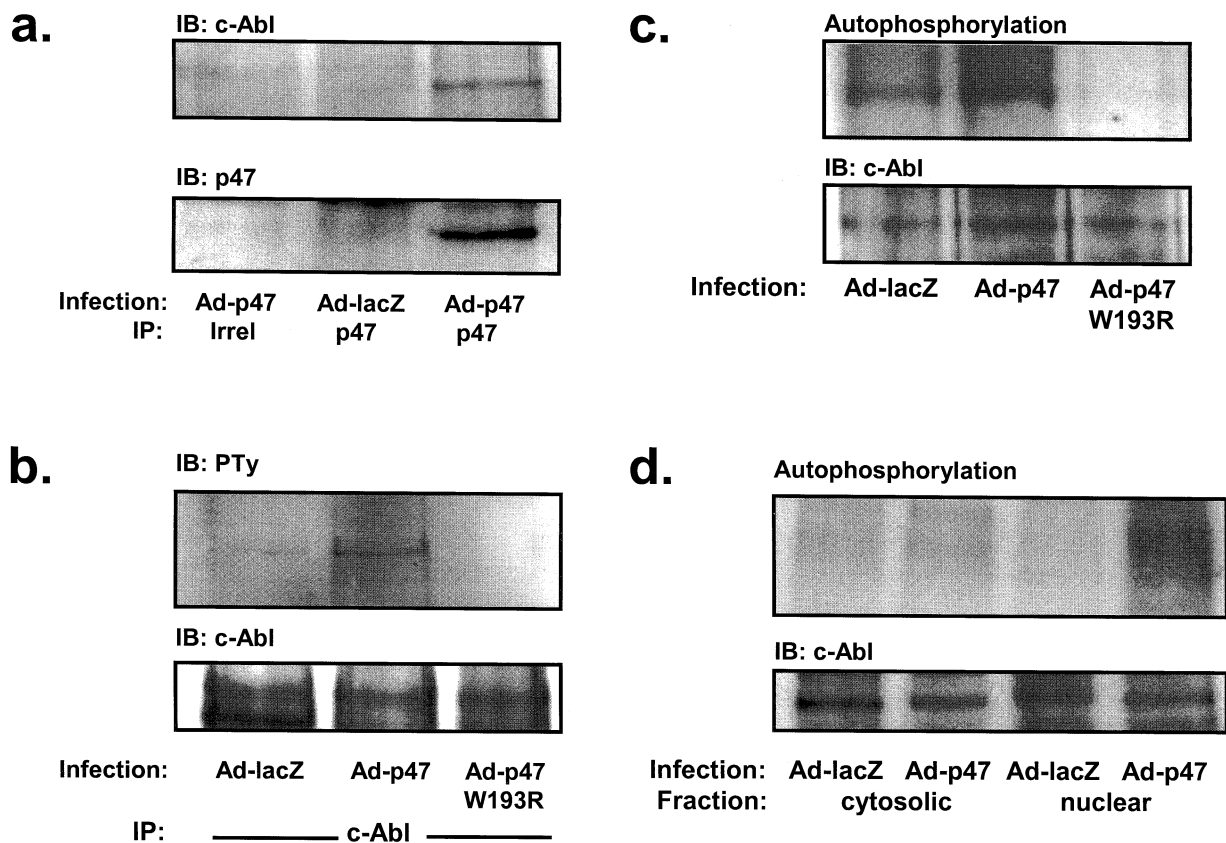


Fig. 4. Overexpression of p47^{phox} activates endogenous c-Abl. a: Endogenous c-Abl from Caco-2 cells was immunoprecipitated with overexpressed p47^{phox} (third lane). c-Abl was not recovered with irrelevant antisera (first lane) or with anti-p47 in the absence of overexpressed p47 (second lane). b: Caco-2 cells were infected with the indicated viruses for 24 h, and endogenous c-Abl was immunoprecipitated. Infection with Ad-p47 increased, whereas infection with Ad-p47(W193R) decreased, tyrosine phosphorylation of c-Abl, compared with Ad-lacZ-infected cells. Representative of two experiments. c,d: Activation of c-Abl was assessed as autophosphorylation of immunoprecipitated c-Abl, 24 h after infection with the indicated viruses. Ad-p47 increased total (c) and nuclear (d) c-Abl activity. Cytosolic c-Abl activity did not change. Representative of three experiments.

mediates cell death independent of p53 [29,30]. This latter c-Abl-dependent death pathway, however, requires intact mismatch-repair capacity, specifically the *MLH1* gene product [29]. Interestingly, the apoptotic influence of p47^{phox} is manifest in SW48 colon cancer cells (Fig. 2b), which are p53wt but deficient in *MLH1* expression, suggesting that neither p53 induction nor mismatch-repair competence are absolutely required for p47-dependent apoptosis. These two death pathways may be duplicative with only one required to trigger apoptosis. Alternatively, p47^{phox} may initiate a novel, c-Abl-dependent death pathway which does not require DNA damage response elements such as p53 and *MLH1*. Such a mechanism may be consistent with the physiologic death of differentiated colonic epithelial cells which normally have not sustained DNA damage, and may explain how the loss of p47^{phox} contributes to tumorigenesis.

From our limited clinical observation, it is not possible to establish a firm causative link between colon tumorigenesis and the loss of expression of p47^{phox}. However, it is noteworthy that genetic deficiency of the phagocyte oxidase (chronic granulomatous disease) due to loss of function mutations in p47^{phox} or gp91^{phox} has been estimated to confer a 13.8-fold increase in the relative risk of acquiring cancer [31], and cells lacking gp91^{phox} are resistant to camptothecin-induced apoptosis [32]. Conversely, the pro-apoptotic effects of p47^{phox}

may also indicate an inseparable proliferative role for the NAD(P)H oxidase. Indeed, a number of oncogenes, typified by *c-myc*, *Ras*, and the viral E1A, initiate tightly coupled proliferative and apoptotic events, the latter of which can

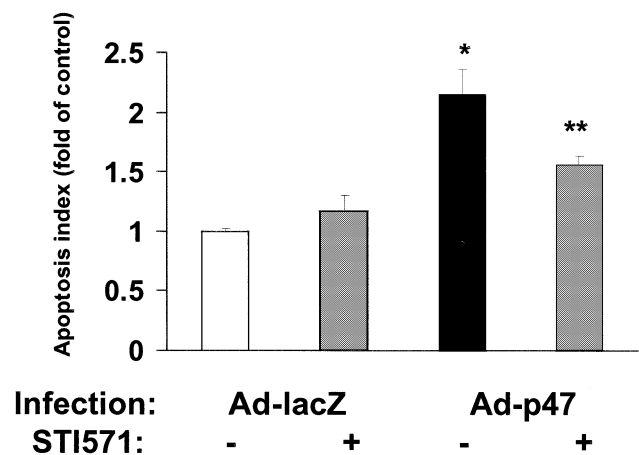


Fig. 5. Caco-2 cells were infected with Ad-lacZ or Ad-p47, and then incubated with STI571 (10 μM) for 24 h. Ad-p47 increased apoptosis compared to Ad-lacZ (**P* < 0.05), and STI571 decreased apoptosis rates in Ad-p47 but not Ad-lacZ-infected cells (***P* < 0.05 compared to Ad-p47 without STI571 treatment).

only be gainsaid by autocrine survival signals [33]. Accordingly, oxidants, ABI-1, and c-Abl all appear capable of mediating both growth and death signals in different contexts.

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