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Induction of FLIP expression by androgens protects prostate cancer cells from TRAIL-mediated apoptosis

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Abstract

BACKGROUND—Prostate tumors initially regress in response to androgen-ablation therapy. However, most cancers eventually relapse with an androgen-depletion-independent (ADI) phenotype that is often more aggressive than the original androgen-dependent (AD) tumor. Importantly, most relapsed tumors still rely upon androgen receptor (AR) activity for proliferation and survival. The cellular Fas/FasL-associated death domain protein-like inhibitory protein (FLIP) inhibits activation of procaspase-8 by death receptor-mediated signaling at the cell surface. In the current study, we examined the androgenic regulation of FLIP and its contribution to protecting prostate cancer cells from death receptor-mediated apoptosis.

METHODS—FLIP expression in tissues from intact and castrated rats as well as androgen-treated prostate cancer cell lines (LNCaP, C4-2, LNCaP-Rf, and DU-145) was monitored *via* Real-Time RT-PCR and immunoblot. Induction of apoptosis by TRAIL, the death receptor ligand, was determined *via* microscopic observation and cell counting of fragmented nuclei following fixation and staining with Hoechst 33285.

RESULTS—FLIP mRNA and protein expression was reduced following castration in multiple rat tissues, including dorsolateral prostate and seminal vesicles. Androgenic induction of FLIP mRNA and protein was observed in isogenic AD LNCaP and ADI LNCaP-Rf cells, but not the isogenic ADI C4-2 cell line. Protection from TRAIL-induced apoptosis by androgen was completely blocked when LNCaP-Rf cells were depleted of endogenous FLIP via siRNA transfection.

CONCLUSIONS—Androgenic protection from TRAIL-induced apoptosis is predominantly via enhanced transcription of FLIP in prostate cancer cells. Loss of androgen-sensitivity in ADI prostate cancer cells highlights this pathway as a potential target for future therapy of prostate cancer.

Keywords

Androgen Receptor; TRAIL; apoptosis; androgen depletion independent (ADI)

Introduction

Prostate cancer is the most frequently diagnosed male cancer and the second leading cause of male cancer mortality [1]. Most prostate tumors are dependent initially on androgens for proliferation and survival. First-line systemic treatment for cancers that cannot be surgically resected is usually androgen-ablation, which blocks the production of androgens or inhibits

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their binding to the androgen receptor (AR) [2]. The AR is a member of the nuclear receptor superfamily of transcription factors and is the key mediator of androgen effects in target tissues. Initially, prostate cancer usually responds to androgen ablation therapy, however most cancers eventually relapse with a hormone-refractory, or androgen-depletion-independent (ADI) phenotype. No treatment currently exists for this fatal stage of the disease.

Despite the apparent androgen-independence of ADI prostate cancer, the AR is still expressed and is functionally active in prostate tumor cells [3]. Transactivation of the AR in ADI prostate cancer cells has been postulated to occur through a number of non-mutually-exclusive mechanisms such as AR mutation or amplification, altered expression of co-regulator proteins, or activation of other cell survival or proliferation pathways [4]. Aberrant AR activation in ADI prostate cancer cells results in induction or repression of androgen-regulated genes, which promotes disease proliferation and survival. Recent efforts to catalog the transcripts regulated by androgens in human tissues and animal models, both normal and neoplastic, have generated an extensive panel of factors involved in a number of important cellular pathways [5]. Of particular importance to prostate cancer are those that regulate cellular survival and progression. Thorough characterization of individual androgen-regulated genes may provide important potential targets that could be exploited to create more effective treatments for prostate cancer.

The cellular FLICE-like inhibitory protein, FLIP (cFLAR, I-FLICE, FLAME-1, CASPER, CLARP, MRIT, Usurpin), is an inhibitor of death-receptor-mediated apoptosis [6–8]. FLIP is expressed in three different isoforms that are found at a single locus and are the result of alternative splicing [8,9]. All three isoforms contain two death-effector domains (DED), which bind to DED-containing adaptor proteins such as FADD at the death-inducing signaling complex (DISC). Binding of FLIP prevents the autocatalytic cleavage of caspase zymogens, thus inhibiting their activation and apoptosis. FLIP has been implicated in a number of cancers, and has been shown to be regulated both at the transcriptional and post-translational level [10]. Several studies have recently examined the potential involvement of FLIP in prostate cancer. These studies have shown that the FLIP promoter contains four androgen response elements and FLIP mRNA is androgen regulated in androgen-dependent LNCaP cells [11]. Moreover, overexpression of FLIP in an LNCaP xenograft mouse model accelerated tumor growth after castration [11].

In order to more fully understand the role of FLIP in prostate cancer development and progression, we sought a more thorough examination of its regulation in normal and malignant prostate tissue. We focused on the regulation of the long isoform of FLIP (55 kD), because previous reports have shown that it is the predominant isoform expressed in prostate cancer cells [12,13]. In the current study, using a rat model, we demonstrate that FLIP mRNA and protein levels were generally decreased in androgen-responsive tissues following castration, and restored by testosterone administration. Using three isogenic prostate cancer cell lines, we also found that FLIP androgen-responsiveness was altered in ADI prostate cancer cells. Importantly, in the absence of androgens, basal expression of FLIP did not require the AR in ADI prostate cancer cell lines. We further show that androgen-mediated protection from TRAIL (TNF-related apoptosis-inducing ligand)-induced apoptosis is due largely to FLIP upregulation. Cells depleted of FLIP via siRNA knockdown remained apoptotic compared to control transfected cells, which were resistant to the death ligand after androgen treatment. Overall, our data indicate that the androgen-regulation of FLIP is a primary mediator of androgenic protection from TRAIL-mediated apoptosis and suggest that strategies aimed at reducing FLIP expression are plausible for future therapies for ADI prostate cancer.

Materials and Methods

Animals

Male Wistar rats were divided into three experimental groups to determine the effect of androgen deprivation and subsequent testosterone treatment on gene expression in multiple tissues as described by Heemers *et al*. [14]. Briefly, experimental groups contained 4 rats each. Group I was sham-operated. Group II and III rats were castrated. Following surgery group II rats received daily subcutaneous vehicle injections. Group III rats received daily injections of a mixture of testosterone and testosterone propionate (0.25 mg each, dissolved in 10 μl EtOH and mixed with 90 μl olive oil). Treatments continued daily for 4 days, at which point all rats were anaesthetized and exsanguinated. Tissues were excised, weighed, and immediately frozen for subsequent RNA and protein analysis.

Cell Lines and Culture Conditions

LNCaP and DU-145 cells were purchased from the American Type Tissue Collection (Manassas, VA). C4-2 cells were purchased from UroCor (Oklahoma City, OK). LNCaP-Rf cells were generated in the Tindall lab as previously described [15]. LNCaP and C4-2 cells were maintained in RPMI 1640 media supplemented with 9% fetal bovine serum and 0.01% antibiotic-antimycotic (Invitrogen, Carlsbad, CA). DU-145 cells were maintained in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 9% fetal bovine serum and 0.1% antibiotic-antimycotic. LNCaP-Rf cells were maintained in phenol-red-free RPMI supplemented with 9% charcoal-stripped (steroid-depleted) serum and 0.01% antibiotic-antimycotic. All cells were maintained at 37°C and 5% CO_2.

For androgen response experiments, cells were seeded in 9% charcoal-stripped serum and grown for 48 h. At time of treatment, growth media was replaced with 9% charcoal-stripped serum media containing 1 nM mibolerone (MIB) (BIOMOL, Plymouth Meeting, PA) or an equivalent amount of EtOH as vehicle control. Cells were cultured for the indicated amount of time and then harvested.

Transfection of siRNA

The SmartPool siRNA duplexes for FLIP, AR, and a non-specific control (CTRL)were purchased from Dharmacon (Waltham, MA). For LNCaP and LNCaP-Rf cells, approximately 3×10^6 cells were suspended in 375 μl 9% charcoal-stripped serum in appropriate media and mixed with 240 pmol of the RNA duplex. The cell/siRNA mixture was transferred to a cuvette with a 4 mm gap width and electroporated with a 305 V electrical pulse for 10 ms using a BTX ElectroSquare electroporator. For siRNA transfection in C4-2 cells, 1×10^6 cells were seeded in 6 cm plates in media containing 9% charcoal-stripped serum and were transfected 24 h later with siRNA as per the SuperFect protocol (Qiagen, Valencia, CA). Half of each electroporation was seeded onto a 6 cm plate containing 6 mL charcoal-stripped serum media and incubated 24 h before indicated treatments.

Real-Time Reverse-Transcription PCR (Real-Time RT-PCR)

Total RNA was isolated from cells via acid-guanidinium phenol/chloroform extraction [16]. To eliminate genomic DNA, 6 μg total RNA was DNase-digested using DNase I (Roche, Basel, Switzerland). Half of the DNase-digested RNA was used in cDNA synthesis using the SuperScriptIII First-Strand cDNA Synthesis Kit (Invitrogen). Quantitative Real-Time PCR was performed using SYBRgreen PCR mastermix (ABI Biosystems, Foster City, CA) following manufacturer instructions on an ABI Prism 7900 HT instrument (ABI Biosystems). Human $FLIP_{total}$ primers were FW: AAGCTGTCTGTCGGGGACTT and RV: GGTGGGTCTCCACAGCTTTT. Human $FLIP_{long}$ primers were FW:

CTTGGCCAATTTGCCTGTAT and RV: CCCATGAACATCCTCCTGAT. Human PSA primers were FW: AGGCCTTCCCTGTACACCAA and RV: GTCTTGGCCTGGTCATTTCC. Human GAPDH primers were purchased from ABI Biosystems. Rat FLIP primers were FW: TCGTGGATCTCCATGTTGAA and RV: AGGCTTTGGGTTTTCATGTG. Rat 18s primers were FW: CATGGCCGTTCTTAGTTGGT and RV: GAACGCCACTTGTCCCTCTA. The fold-change in expression levels were determined by a comparative C_T method using the formula $2^{-\Delta\Delta C_T}$; where C_T is the threshold cycle of amplification. All samples were normalized to internal GAPDH or r18s controls.

Immunoblotting and Densitometry

Total protein lysates were collected in an SDS lysis/loading buffer (65 mM Tris-HCl (pH 7.0), 2% (w/v) SDS, 5% β-mercaptoethanol, 10% (v/v) glycerol, and 0.5% (w/v) bromophenol blue). Equal protein amounts (typically 30 μg per lane) were separated in 10% NuPage gels (Invitrogen), followed by transfer to nitrocellulose membranes and membrane blocking. Primary antibodies were diluted according to manufacturer instructions. Primary antibodies used were anti-AR (N-20, Santa CruzBiotechnology, Santa Cruz, CA), anti-β-actin (Cell Signaling Technology, Danvers, MA), anti-BID (Cell Signaling Technology), anti-Erk2 (D-2, Santa Cruz Biotechnology), anti-FLIP (Cell Signaling Technology), anti-FLIP (NF6, Alexis Biochemicals, Lausen, Switzerland), anti-phospho Akt (587F11, Cell Signaling Technology), anti-Akt (11E7, Cell Signaling Technology), anti-XIAP (Cell Signaling Technology), and anti-β-tubulin (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated secondary antibodies (GE Healthcare Life Sciences, Piscataway, NJ) were diluted 1:5,000 to 1:10,000. Membranes were incubated in Supersignal West Pico Substrate, Supersignal West Femto Substrate (Pierce, Rockford, IL), or ECL Plus (GE Healthcare Life Sciences) according to manufacturer instructions and exposed to film. Densitometric analysis was performed using ScionImage software (NIH).

Hoechst Staining and Cell Counts

Following experimental treatments, media containing detached, apoptotic cells was aspirated and saved. Adherent cells were removed from plate using trypsin (0.25% Trypsin-EDTA, Invitrogen) and added to the saved media. Cells were washed with PBS and resuspended in 1 mL fixing solution (methanol:acetic acid (3:l v/v)) at room temperature overnight. Cells were pelleted and resuspended in 200 μl of fixing solution. Two drops were transferred to a clean microscope slide, and allowed to air dry for 1 hour. Stain solution (1 μg/mL Hoechst 33258 in glycerol:0.1M Tris pH 1, 1:1 v/v) was dropped onto cells and covered with a coverslip. To quantitate apoptotis, 200 cells per slide were counted using an Axioplan 2 fluorescence microscope (Zeiss, Oberkochen, Germany) under a DAPI fluorescence filter. Data represents three independent experiments with samples analyzed in triplicate.

Results

FLIP mRNA and protein is regulated by androgens in multiple rat tissues

A previous study by Nastiuk *et al.* [17] previously showed regulation of FLIP mRNA by androgens in the rat ventral prostate, but did not examine FLIP protein expression. Therefore, we first examined the regulation of FLIP mRNA and protein by androgens in male Wistar rats. We measured the expression of FLIP protein and mRNA in all three lobes of the rat prostate, as well as other androgen-sensitive tissues such as seminal vesicle and lacrimal glands. Androgen-insensitive tissues such as kidney, liver, and spleen were employed as controls. Three groups of rats were either sham-operated, castrated and given vehicle injections, or castrated and given testosterone injections for four days post-surgery, at which time the rats were sacrificed and organs isolated. As expected, the weights of

androgen-sensitive organs were reduced in castrated rats (data not shown). Similarly, in testosterone-supplemented castrated animals, normal organ weight was maintained (data not shown). As shown in Fig. 1A, FLIP mRNA expression in all androgen-sensitive tissues except seminal vesicles was inhibited by castration (top row). Surprisingly, castration induced FLIP mRNA expression in seminal vesicles, an observation that we cannot explain. Testosterone supplementation blocked castration-mediated effects in all tissues. Interestingly, expression of FLIP mRNA in the kidney and liver was slightly increased upon castration, while FLIP mRNA expression was decreased in the spleen following castration. These data demonstrate that FLIP mRNA is regulated by androgens in both androgen-sensitive and androgen-insensitive tissues.

We were able to detect FLIP protein only in the seminal vesicle and dorsolateral prostate by immunoblot. Therefore, we investigated the effect of androgens on FLIP protein expression in these two tissues (Fig. 1B). Surprisingly, in the seminal vesicle, FLIP protein expression was reduced by castration. This was in contrast to FLIP mRNA induction observed following castration (Fig. 1A). FLIP protein expression in the dorsolateral prostate was also reduced by castration, which matched the mRNA expression profile in this tissue. AR expression was also examined in the seminal vesicle, as well as the dorsolateral and ventral prostates to assess whether AR expression might account for varying FLIP protein and mRNA expression in these tissues (Fig. 2C). Both FLIP and AR expression were highest in the seminal vesicle. In dorsolateral and ventral prostates, expression levels of the AR did not correlate with the level of FLIP mRNA or protein expression. Overall, these data demonstrate that FLIP protein expression is decreased by castration in normal tissues.

Androgenic regulation of FLIP mRNA and protein is altered in ADI cells

Previous work has demonstrated that FLIP mRNA is regulated by androgens in LNCaP cells and that this regulation is mediated through the recruitment of the AR to AREs in the FLIP promoter region [11]. In order to more fully characterize the androgenic regulation of FLIP in prostate cancer cells, we used isogenic cell lines that model the progression of ADI disease *in vitro*. LNCaP cells were used as a model for AD prostate cancer, whereas C4-2 and LNCaP-Rf cells were used as models for ADI prostate cancer [18]. C4-2 cells are ADI and were derived from LNCaP cells that had been serially xenografted through castrated mice [18]. LNCaP-Rf cells are also ADI and were generated by long-term culture of LNCaP cells in the absence of androgens [15]. For our examination of FLIP mRNA expression, we used two sets of real-time RT-PCR primers: one that was specific for the long isoform only and one that recognizes all isoforms. The data obtained from both primer sets was similar in all experiments, indicating that the effect of androgens on FLIP expression in prostate cancer cells represents expression of the large isoform only (data not shown). Quantitative RT-PCR and immunoblots demonstrated that basal expression of both FLIP protein and mRNA were higher in C4-2 cells compared to LNCaP, while expression in LNCaP-Rf cells was similar to LNCaP (Fig. 2A). After 24 h treatment in LNCaP cells, an increase in the expression of FLIP was observed at both the mRNA and protein level at 0.1 nM MIB, and reached a maximum at 10 nM MIB (Fig. 2B). Expression of PSA mRNA was monitored concurrently as a control for effective androgenic activation of AR (Fig. 2B-D, lower panels). Interestingly, the two different ADI lines exhibited differences in FLIP regulation by androgens. In C4-2 cells, the expression of FLIP mRNA and protein remained unchanged in response to MIB dose treatment (Fig. 2C). However, in LNCaP-Rf cells, FLIP mRNA and protein levels were increased after 24 h treatment with 1 nM MIB (Fig. 2D). The lack of an androgenic response observed in C4-2 cells suggests that androgenic regulation of FLIP can become deregulated during the transition of these cells from AD to ADI, possibly contributing to the progression of prostate cancer. The deregulation seen in C4-2 cells might be due to effects from *in vivo* selection, as LNCaP-Rf cells selected *in vitro* behaved more

like the parental LNCaP line (Fig. 2D). Furthermore, the increased levels of FLIP mRNA and protein observed in C4-2 cells suggest that mechanisms which regulate basal expression are altered during the progression to ADI disease.

The AR is important for androgenic regulation of FLIP, but not for basal expression in prostate cancer cells

Because of the differences observed in the androgen-sensitivity in these model cell lines, as well as the high basal expression of FLIP in C4-2 cells, we sought to determine the extent to which the AR was necessary to regulate FLIP expression. First, we examined whether the AR was necessary to regulate FLIP in response to androgens by examining FLIP expression in the AR-null prostate cancer cell line, DU-145 [18]. After treatment with 1 nM MIB for 24 h in charcoal-stripped serum, no change in FLIP mRNA or protein level was observed (Fig. 3A). We also assessed whether androgen had an effect on FLIP expression following siRNA-mediated AR knock-down in LNCaP cells. Androgen treatment induced FLIP expression in control siRNA-transfected cells, but had no effect in cells transfected with AR-targeted siRNAs (Fig. 3B). These data indicate that the AR is required for androgenic upregulation of FLIP.

To determine if the higher basal levels of FLIP observed in C4-2 cells was due to constitutive AR activity, all three cell lines were transfected with siRNA directed against the AR in charcoal-stripped serum. As shown in Fig. 3C, AR knockdown was successful in all three cell lines. In LNCaP and LNCaP-Rf cells, FLIP protein (upper panels) and mRNA (lower panels) remained unchanged in AR-depleted cells compared to mock or control siRNA transfected cells. FLIP mRNA levels were unchanged in siAR-transfected C4-2 cells; however, a small decrease in FLIP protein was observed. Importantly, expression of PSA mRNA was significantly reduced by AR siRNA in all three cell lines. These data suggest that the AR is not necessary for maintaining a basal level of FLIP expression in prostate cancer cells in the absence of androgens.

Androgenic protection from TRAIL-induced apoptosis is mediated largely through FLIP upregulation in prostate cancer cells

TRAIL is a soluble protein ligand that initiates an apoptotic signaling cascade upon binding to receptors DR4 and DR5 present in the cell membrane. The different basal FLIP expression levels observed in Fig. 2A suggested that the LNCaP and derivative cell lines would have varying sensitivities to TRAIL treatment under conditions of androgen ablation. After culturing cells in different media conditions, we found that FLIP expression levels were indeed lowest in all three cell lines when cultured in charcoal-stripped serum (compared to whole serum and charcoal-stripped serum with MIB) (Fig. 4A). The lower level of FLIP observed in the three cell lines when cultured in charcoal-stripped serum suggests that the cells would be most sensitive to TRAIL treatment under this condition. To test the hypothesis that the varying levels of FLIP expression in the three cell lines as seen in Fig. 2A is predictive of their comparative sensitivity to TRAIL, the cells were grown in charcoal-stripped serum and treated with varying doses of TRAIL for 24 h. Hoechst staining followed by counting of fragmented nuclei revealed that FLIP expression was not entirely predictive of TRAIL sensitivity (Fig. 4B). LNCaP-Rf cells, which expressed amounts of FLIP protein and mRNA comparable to LNCaP cells were the most sensitive to treatment, achieving approximately 50 percent cell death with 10 ng/mL TRAIL. LNCaP cells were highly resistant to TRAIL treatment, as has been described [12,19]. C4-2 cells expressed higher levels of FLIP than LNCaP and LNCaP-Rf, but displayed TRAIL sensitivity intermediate to LNCaP and LNCaP-Rf. Due to the inability to correlate FLIP expression with cellular response to TRAIL, we sought an explanation for this difference by examining the expression of a panel of proteins that regulate cell survival and apoptosis (Fig. 4C).

LNCaP cells have constitutively active Akt due to PTEN inactivation, rendering them resistant to death receptor mediated apoptosis [20,21]. In the three cell lines maintained in charcoal-stripped serum, active, Ser473-phosphorylated Akt was present in LNCaP as well as C4-2 cells, but not in LNCaP-Rf cells (Fig. 4C). However, overall Akt expression levels were increased in C4-2 and LNCaP-Rf compared to LNCaP. Also, increased expression of full-length Bcl-2 interacting protein (Bid), which can propagate apoptotic signaling, was expressed at a higher level in the ADI cell lines. Expression of the X-linked inhibitor of apoptosis protein (XIAP), an inhibitor of caspases 3, 7, and 9, was similar in all three lines. These data suggest that while FLIP expression is different in these cell lines, other intrinsic changes, such as reduced levels of activated Akt, are likely to be important for determining TRAIL sensitivity.

Next, we tested whether androgen treatment would alter the sensitivity of prostate cancer cell lines to TRAIL mediated apoptosis. In LNCaP-Rf cells, apoptosis was significantly reduced by pre-treatment with 1 nM MIB for 8 h prior to death ligand treatment (Fig. 5A). In C4-2 cells, no effect of MIB pre-treatment was observed on TRAIL sensitivity (Fig. 5A), which is consistent with the inability of C4-2 cells to regulate FLIP in response to androgen treatment (Fig. 2C). In agreement with our previous data, apoptosis was not induced by TRAIL in LNCaP cells regardless of androgen treatment.

Because androgen has been shown to regulate the expression of a number of apoptotic regulators [5], we were interested to determine whether FLIP is a key effector of androgen-signaled protection from TRAIL. LNCaP-Rf cells maintained in charcoal-stripped serum were electroporated with FLIP-directed siRNA and subsequently treated with MIB with or without TRAIL. FLIP protein was decreased by siRNA at 48 h after transfection, the experimental endpoint of the assay (Fig. 5D). After 16 h TRAIL treatment, (Fig. 5C Hoechst images and 5B quantitation), cells transfected with siFLIP had approximately 15% more apoptotic nuclei following TRAIL treatment than control siRNA-transfected cells, indicating that FLIP knock-down sensitized the cells to the death ligand. In mock and control siRNA-transfected cells, androgens blocked TRAIL-mediated apoptosis by approximately 50%. However, no androgen-mediated protection from apoptosis was observed in siFLIP-transfected cells. These data indicate that FLIP upregulation is critical for androgen-mediated protection from death-receptor mediated apoptosis.

Discussion

In normal and neoplastic prostate cells, androgens stimulate AR-mediated transcription of factors involved in differentiation, proliferation, and survival. Androgen signaling and subsequent AR activity have been demonstrated to be necessary for early prostate cancer development [5]. ADI prostate cancer cells remain dependent upon the AR for survival, which suggests that key transcriptional targets of the AR are important in this later stage of the disease. This study investigated the role of FLIP regulation by androgens. As a homolog to procaspase-8, the long isoform of FLIP is able to competitively bind to the same adaptor proteins present on the intracellular surface of the death-inducing signaling complex (DISC) to inhibit autocatalytic cleavage of adjacent procaspase-8 molecules and inhibit downstream apoptotic signaling. Initiation of DISC signaling can come from a number of soluble, extracellular death ligands, including FasL, TNF-α and TRAIL.

Previous studies have demonstrated that FLIP is an androgen-regulated gene, however, the functional aspects of this regulation have not been addressed. Nastiuk *et al.* previously described FLIP mRNA to be regulated by androgens in the ventral prostate [17], which is supported by our data. Our data further extends these findings and shows that FLIP mRNA is regulated by androgens in all three lobes of the prostate, as well as the lacrimal gland,

seminal vesicles, kidney, liver, and spleen. The regulation of the protein in these normal tissues also reflected the mRNA regulation. Surprisingly, regulation of FLIP mRNA and protein did not correlate with each other in the seminal vesicles, which indicates that other factors are also involved in regulating FLIP expression in this tissue. Our data showing androgenic regulation of FLIP in LNCaP cells (Fig. 2B) agrees with data previously presented by Gao *et al.* that the AR directly regulates FLIP transcript in response to androgen [11]. Further, we observed that the regulation of FLIP by androgens is altered in ADI cells. FLIP mRNA and protein expression increased in response to androgens in LNCaP-Rf cells in a similar manner to parental LNCaP cells (Fig. 2D). However, no change in FLIP mRNA or protein was observed in C4-2 cells following androgen treatment (Fig. 2C). Overall, our data demonstrate that FLIP mRNA and protein expression is regulated by androgens in both normal and neoplastic prostate tissue, and that this regulation is lost in the C4-2 model of ADI prostate cancer.

This study provides the first examination of how androgens protect cells from death-receptor mediated apoptosis *via* regulation of FLIP expression. We focused on TRAIL-induced apoptosis as this pathway has been described to be active and sensitive to androgen in some prostate cancer cells [22]. As has been previously demonstrated, we observed that LNCaPs were insensitive to TRAIL treatment [12,13,19]. The LNCaP line, like many prostate cancers, has been described to contain a nonsense PTEN mutation [20,21,23]. As such, membrane phosphatidylinositol-3,4,5-triphosphate levels remain high and persistent activation of downstream Akt occurs, promoting cell survival. Accordingly, we were unable to detect PTEN protein in any of the three cell lines. Surprisingly, we were only able to detect phosphorylated Akt in LNCaP and C4-2 (Fig. 4B). Therefore, we observed increasieng sensitivity to TRAIL in each of the isogenic ADI lines, LNCaP-Rf and C4-2, which correlated with their comparative expression levels of FLIP (Fig. 2A) and active Akt (Fig. 4B). The LNCaP-Rf cells were the most sensitive to TRAIL (Fig. 4A), and their sensitivity was decreased by androgen pretreatment (Fig. 5A). Importantly, our data demonstrate that the protective effect induced by androgens is primarily mediated *via* FLIP. For example, knockdown of FLIP expression in the presence of androgens failed to rescue any cells from apoptosis (Fig. 5B). C4-2 cells were slightly sensitive to TRAIL (Fig. 4A), which correlated with the observed higher basal FLIP expression compared to LNCaP-Rf (Fig. 2A). The reason for this higher basal FLIP expression is unclear, however knockdown of the AR indicates that it is not required to sustain the basal level of FLIP in C4-2 cells. The observation that FLIP is not regulated by androgens, but is sustained at a higher basal level in an AR-independent manner in C4-2 cells compared to LNCaP and LNCaP-Rf suggests that the AR requirement to increase FLIP expression is bypassed in these cells. Many mechanisms exist that might allow for this possibility, including signaling from other pathways and alteration in expression or activity of AR co-regulators [4]. Further studies are warranted to examine if this possibility is true and to identify which pathways are important for bypassing the AR requirement to regulate FLIP in ADI cells.

Based on our findings that FLIP is a critical mediator of androgen-mediated protection from TRAIL-induced apoptosis, we propose that a combination treatment of androgen ablation plus TRAIL administration could be a plausible therapy for a subset of ADI prostate cancer. Currently, recombinant TRAIL injection is being investigated in mouse models as a treatment for a number of cancers, including colon, breast, multiple myeloma, glioma, and pancreatic andenocarcinoma [24]. TRAIL is highly selective for tumor cells, and is well-tolerated systemically [24]. Our examination of TRAIL sensitivity in ADI LNCaP-Rf cells suggests that for some patients with recurrent ADI disease, continued blockade of androgen signaling would maintain low amounts of FLIP expression, thus enhancing the therapeutic window for TRAIL treatment. For ADI tumors that bypass the AR requirement for FLIP regulation, as observed for C4-2 cells, this kind of approach would not be feasible.

In summary, our data point toward a primary involvement of FLIP in androgen-mediated apoptosis evasion in prostate cancer cells. This indicates that a sustained androgen ablation therapy coupled with TRAIL, or other death ligand, administration might be an effective treatment for the subset of ADI cancers that retain the ability to regulate FLIP expression in response to androgens. Elucidation of those mechanisms that allow for the bypass of the AR to regulate FLIP expression will permit the design of therapeutic strategies to reduce FLIP expression in ADI tumors and thus make them more susceptible to death ligand-based treatments.

Conclusions

FLIP is an androgen-regulated gene whose regulation by androgen is evident both *in vivo* and *in vitro*. While the AR is not required for basal FLIP expression in prostate cancer cells, it is required for androgen-mediated upregulation of FLIP mRNA. During the transition from an androgen-dependent tumor to and ADI phenotype, cells may lose the ability to regulate FLIP in response to androgens. The ability of androgens to protect cells from TRAIL-induced apoptosis is mediated primarily through FLIP upregulation. FLIP remains an interesting target for future study and may provide options for treatment of ADI disease.

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References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer Statistics, 2007. CA Cancer J Clin. 2007; 57(1):43–66. [PubMed: 17237035]
2. Miyamoto H, Messing EM, Chang C. Androgen deprivation therapy for prostate cancer: Current status and future prospects. The Prostate. 2004; 61(4):332–353. [PubMed: 15389811]
3. Santos AF, Huang H, Tindall DJ. The androgen receptor: a potential target for therapy of prostate cancer. Steroids. 2004; 69(2):79–85. [PubMed: 15013685]
4. Debes JD, Tindall DJ. Mechanisms of Androgen-Refractory Prostate Cancer. New England Journal of Medicine. 2004; 351(15):1488–1490. [PubMed: 15470210]
5. Dehm SM, Tindall DJ. Molecular regulation of androgen action in prostate cancer. Journal of Cellular Biochemistry. 2006; 99(2):333–344. [PubMed: 16518832]
6. Rasper DM, Vaillancourt JP, Hadano S, Houtzager VM, Seiden I, Keen SL, Tawa P, Xanthoudakis S, Nasir J, Martindale D, Koop BF, Peterson EP, Thornberry NA, Huang J, MacPherson DP, Black SC, Hornung F, Lenardo MJ, Hayden MR, Roy S, Nicholson DW. Cell Death Attenuation by "Usurpin", a Mammalian DED-Caspase Homologue That Precludes Caspase-8 Recruitment and Activation by the CD-95 (Fas, APO-1) Receptor Complex. Cell Death Diff. 1998; 5:271–288.
7. Hu S, Vincenz C, Ni J, Gentz R, Dixit VM. I-FLICE, a Novel Inhibitor of Tumor Necrosis Factor Receptor-1- and CD-95-induced Apoptosis. J Biol Chem. 1997; 272(28):17255–17257. [PubMed: 9211860]
8. Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer J-L, Schroter M, Burns K, Mattmann C, Rimoldi D, French LE, Tschopp J. Inhibition of death receptor signals by cellular FLIP. Nature. 1997; 388(6638):190–195. [PubMed: 9217161]
9. Golks A, Brenner D, Fritsch C, Krammer PH, Lavrik IN. c-FLIPR, a New Regulator of Death Receptor-induced Apoptosis. J Biol Chem. 2005; 280(15):14507–14513. [PubMed: 15701649]
10. Kataoka T. The caspase-8 modulator c-FLIP. Crit Rev Immunol. 2005; 25(1):31–58. [PubMed: 15833082]

11. Gao S, Lee P, Wang H, Gerald W, Adler M, Zhang L, Wang Y-F, Wang Z. The Androgen Receptor Directly Targets the Cellular Fas/FasL-Associated Death Domain Protein-Like Inhibitory Protein Gene to Promote the Androgen-Independent Growth of Prostate Cancer Cells. Mol Endocrinol. 2005; 19(7):1792–1802. [PubMed: 15731171]
12. Zhang X, Jin T-G, Yang H, DeWolf WC, Khosravi-Far R, Olumi AF. Persistent c-FLIP(L) Expression Is Necessary and Sufficient to Maintain Resistance to Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand-Mediated Apoptosis in Prostate Cancer. Cancer Res. 2004; 64(19): 7086–7091. [PubMed: 15466204]
13. Hesry V, Claire P-P, Marion T, Ludovic T, Bernard J, Patard J-J, Thierry G. Sensitivity of prostate cells to TRAIL-induced apoptosis increases with tumor progression: DR5 and caspase 8 are key players. The Prostate. 2006; 66(9):987–995. [PubMed: 16541419]
14. Heemers H, Vanderhoydonc F, Roskams T, Shechter I, Heyns W, Verhoeven G, Swinnen JV. Androgens stimulate coordinated lipogenic gene expression in normal target tissues in vivo. Molecular and Cellular Endocrinology. 2003; 205(1–2):21–31. [PubMed: 12890564]
15. Zegarra–Moro OL, Schmidt LJ, Huang H, Tindall DJ. Disruption of Androgen Receptor Function Inhibits Proliferation of Androgen-refractory Prostate Cancer Cells. Cancer Res. 2002; 62(4): 1008–1013. [PubMed: 11861374]
16. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 1987; 162(1):156–159. [PubMed: 2440339]
17. Nastiuk KL, Kim JW, Mann M, Krolewski JJ. Androgen regulation of FLICE-like inhibitory protein gene expression in the rat prostate. Journal of Cellular Physiology. 2003; 196(2):386–393. [PubMed: 12811833]
18. Sobel RE, Sadar MD. Cell Lines Used In Prostate Cancer Research: A Compendium of Old And New Lines--Part 1. The Journal of Urology. 2005; 173(2):342–359. [PubMed: 15643172]
19. Kang J, Bu J, Hao Y, Chen F. Subtoxic concentration of doxorubicin enhances TRAIL-induced apoptosis in human prostate cancer cell line. LNCaP. 2005; 8(3):274–279.
20. Nesterov A, Lu X, Johnson M, Miller GJ, Ivashchenko Y, Kraft AS. Elevated Akt Activity Protects the Prostate Cancer Cell Line LNCaP from TRAIL-induced Apoptosis. J Biol Chem. 2001; 276(14):10767–10774. [PubMed: 11278284]
21. Chen X, Thakkar H, Tyan F, Gim S, Robinson H, Lee C, Pandey S, Nwokorie C, Onwudiwe N, Srivastava R. Constitutively active Akt is an important regulator of TRAIL sensitivity in prostate cancer. Oncogene. 2001; 20:6073–6083. [PubMed: 11593415]
22. Guseva N, Taghiyev A, Rokhlin O, Cohen M. Death receptor-induced cell death in prostate cancer. Journal of Cellular Biochemistry. 2004; 91(1):70–99. [PubMed: 14689583]
23. Vlietstra RJ, van Alewijk DCJG, Hermans KGL, van Steenbrugge GJ, Trapman J. Frequent Inactivation of PTEN in Prostate Cancer Cell Lines and Xenografts. Cancer Res. 1998; 58(13): 2720–2723. [PubMed: 9661880]
24. Merino D, Lalaoui N, Morizot A, Solary E, Micheau O. TRAIL in cancer therapy: present and future challenges. Expert Opinion on Therapeutic Targets. 2007; 11(10):1299–1314. [PubMed: 17907960]

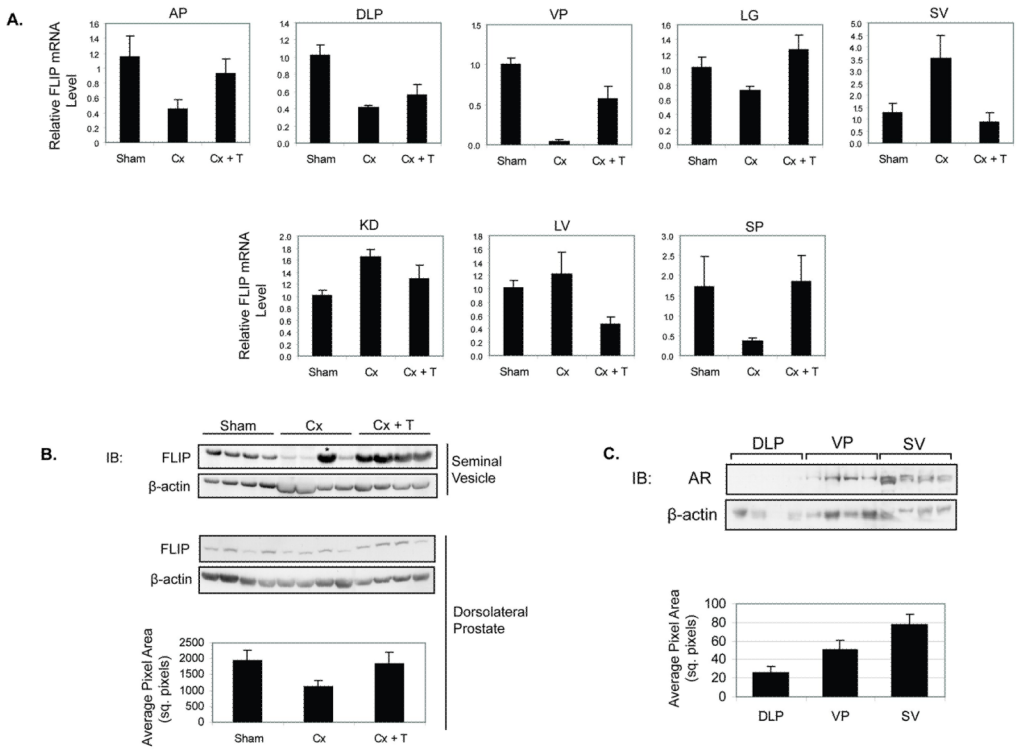

Figure 1.

Androgenic manipulation regulates FLIP expression *in vivo*. (A) Examination of FLIP mRNA expression via real-time RT-PCR. Each experimental group contains 4 animals, with each sample analyzed in triplicate. Groups: sham-operated (Sham), castrated with vehicle injections (Cx), and castrated with testosterone/testosterone propionate injections (Cx + T). Top row graphs represent known androgen-regulated tissues: anterior prostate (AP), dorsolateral prostate (DLP), ventral prostate (VP), lacrimal gland (LG), and seminal vesicle (SV). Bottom row graphs represent androgen-independent tissues: kidney (KD), liver (LV), and spleen (SP). FLIP expression was normalized to internal r18s RNA, and data is presented as relative FLIP mRNA level, normalizing Sham to 1. Data represent the mean ± S.E. from four animals, each analyzed in triplicate. (B) Immunoblot analysis of FLIP expression in tissue lysates from seminal vesicle (top) and dorsolateral prostate (bottom). (*) in the FLIP seminal vesicle blot indicates one animal that had high FLIP expression compared to others in the Cx group, and was believed to be due to animal variation. The dorsolateral prostate FLIP blot was further analyzed by densitometric calculation (below). (C) Immunoblot analysis of AR expression in lysates of dorsolateral prostate, ventral prostate, and seminal vesicle of sham-operated rats. The blot was subjected to densitometric analysis (lower panel). Each bar represents the average pixels for the indicated group, normalized to β-actin expression. Error bars represent ± S.E.

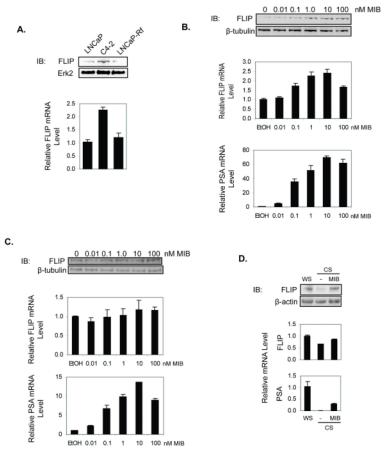

Figure 2.

Androgen regulation of FLIP is variable in ADI prostate cancer cells. Cells were cultured in 9% charcoal-stripped serum for 48 h prior to treatment. Immunoblot data is represented in the upper panels, real-time RT-PCR data is presented in the bar graphs below. Real-time RT-PCR data is expressed as relative mRNA expression level, and values for of one of the untreated samples are arbitrarily set at 1 unless specified otherwise. Data represent the mean ± S.E. from three independent experiments, each performed in triplicate and normalized to an internal GAPDH control. (A) Basal FLIP protein and mRNA expression in prostate cancer cell lines. Expression of FLIP in one untreated sample of LNCaP cells was arbitrarily set at 1. (B) 24h Mibolerone (MIB) dose response in AD LNCaP cells. (C) 24 h MIB dose response in ADI C4-2 cells. (D) Androgen manipulation in ADI LNCaP-Rf cells. WS, whole serum; CS, charcoal-stripped serum; MIB, 1 nM MIB treatment for 24 h.

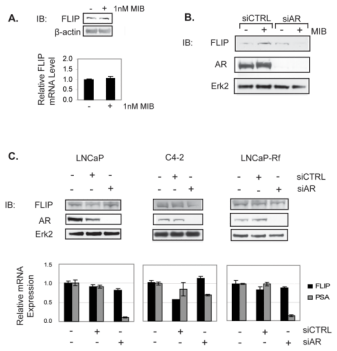

Figure 3.

The AR is required for androgen-induced, but not basal FLIP expression in prostate cancer cells. Cells were cultured in 9% charcoal-stripped serum. Immunoblot data is represented in the upper panels, real-time RT-PCR data is presented in the graphs below. Real-time RT-PCR data is expressed as relative mRNA expression level, and values for one untreated sample is arbitrarily set at 1 unless specified otherwise. Data represent the mean ± S.E. from three independent experiments, each performed in triplicate and normalized to an internal GAPDH control. (A) AR-null DU-145 cells do not regulate FLIP in response to androgen. Cells were treated with EtOH vehicle (−) or 1 nM MIB (+) for 24 h. (B) LNCaP cells with decreased AR expression do not display increased FLIP expression following androgen treatment. Cells were cultured in charcoal-stripped serum media, transfected with a non-targeting control siRNA (siCTRL) or siRNA specific for the AR (siAR), and treated with EtOH vehicle (−) or 1nM MIB (+) for 24h. Cells were harvested 72 h after transfection. (C) LNCaP and isogenic ADI sublines do not show changes in basal FLIP mRNA or protein following AR knock-down. Cells were cultured and transfection as in (B).

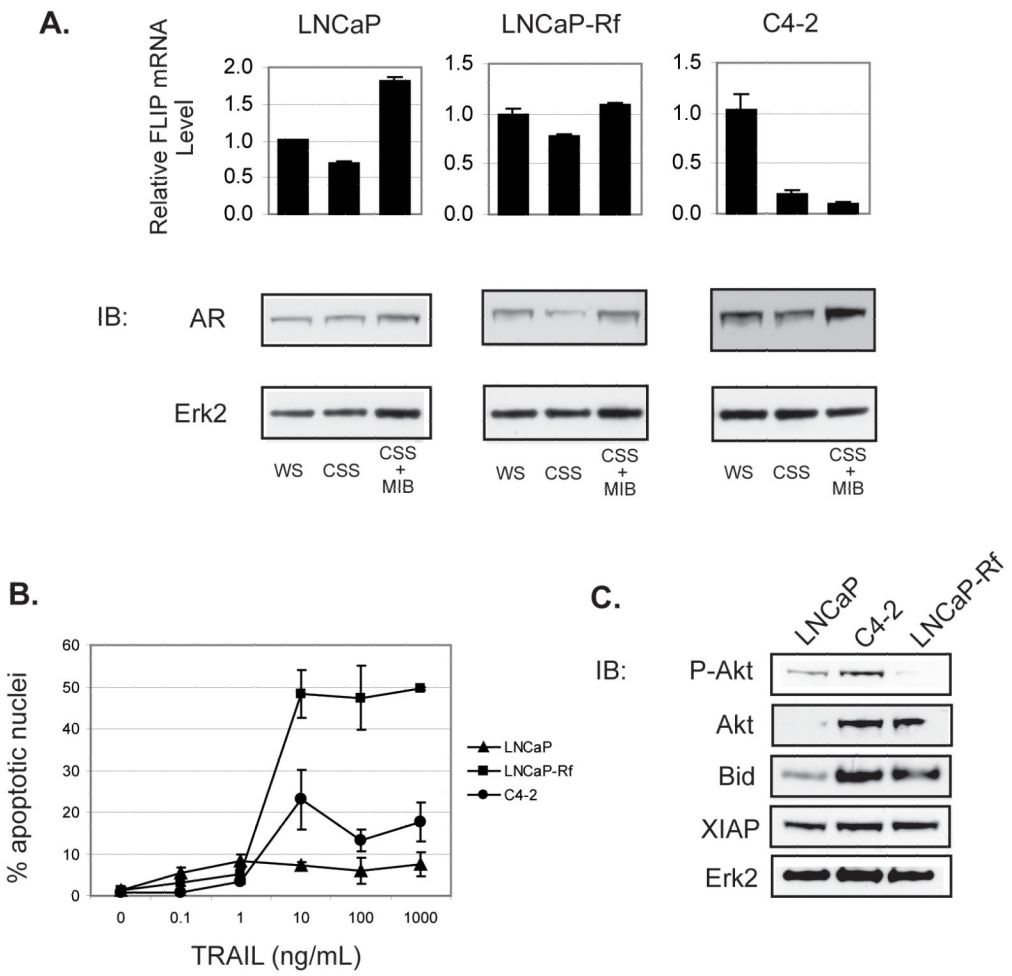

Figure 4.

LNCaP-Rf cells are most sensitive to TRAIL-mediated apoptosis compared to other isogenic prostate cancer cells. (A) Cells were cultured in whole serum (WS), charcoal-stripped serum (CSS), or CSS and treated with MIB for 24h (CSS+MIB). FLIP mRNA levels were analyzed via real-time PCR (upper panels). Values for WS-cultured cells were arbitrarily set at 1 for each cell line. Data is normalized to an internal GAPDH control and represent the mean ± S.E. from samples analyzed in triplicate. Lower panels represent immunoblot analysis of whole cell lysates probed for AR and Erk2. (B) Cells were grown for at least 48 h in charcoal-stripped serum media before treatment with the indicated doses of TRAIL. Cells were fixed, applied to slides, and stained with Hoechst 33258. Nuclei were counted and apoptotic nuclei represented as the mean percentage ± S.E. from three independent experiments, performed in duplicate. (C) Protein lysates from cells cultured for at least 72 h in charcoal-stripped serum media were analyzed for indicated cell survival factors and apoptosis regulators via immunoblot.

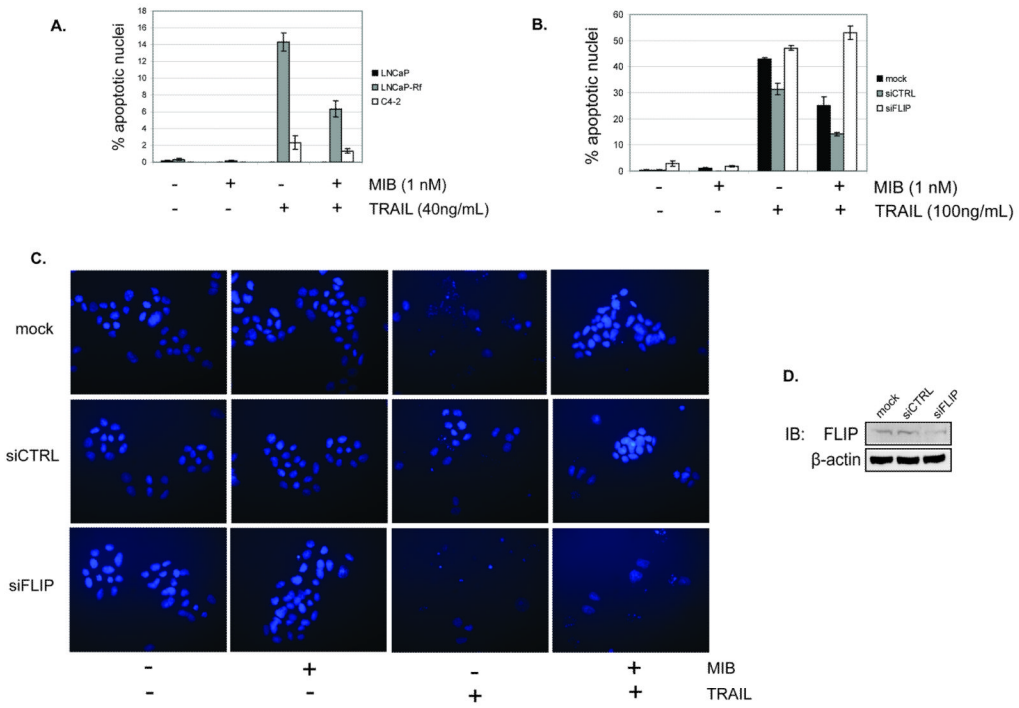

Figure 5.

Androgen-induced protection from apoptosis is largely mediated through FLIP upregulation. (A) Pre-treatment with androgen protects cells from TRAIL-mediated apoptosis. Cells were grown in charcoal-stripped serum for 48 h, and treated with EtOH vehicle (−) or 1 nM MIB for 8 h prior to TRAIL treatment (40 ng/mL) for 16 h. Cells were collected, fixed, and stained with Hoechst 33258 for nuclei counting. Data is presented as the mean apoptotic nuclei percentage ± S.E. from three independent experiments, performed in triplicate. (B) LNCaP-Rf cells were transfected with mock, non-targeting control siRNA (siCTRL), or siRNA targeted to FLIP (siFLIP) and seeded in charcoal-stripped serum media. 24 h later cells were treated with EtOH vehicle (−) or 1 nM MIB for 8 h prior to TRAIL treatment (100 ng/mL) for 16 h. Cells were collected, fixed, and stained with Hoechst 33258 for nuclei counting. Data is presented as the mean apoptotic nuclei percentage ± S.E. from three independent experiments, performed in triplicate. (C) Hoechst-stained DAPI images from cells quantified in (B). (D) Whole cell protein lysates from untreated, transfected LNCaP-Rf cells, 48 h after transfection.