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Five of six prostate cancer cell lines undergo apoptosis when incubated with TRAIL. Cell viability curves of six prostate cancer cell lines using varying amounts of TRAIL protein were completed. Prostate cancer cell lines Alva 31, PC-3 and DU 145 are highly sensitive to apoptosis induced by TRAIL, while the cell lines TSU-pr1 and JCA-1 are moderately sensitive, and the LNCaP cell line is resistant. Protein synthesis inhibitor cycloheximide and AKT inhibitors wortmannin and LY294002 enhance the apoptotic effect of TRAIL. The potential mechanism for resistance to TRAIL-induced apoptosis in prostate cancer cells was examined. No correlation was found between the sensitivity of cells to TRAIL and the expression of TRAIL receptors DR4 and DR5, decoy receptors for TRAIL DcR1 and DcR2, or apoptosis inhibitory protein cFLip. TRAIL-induced apoptotic signaling in resistant cell line LNCaP is blocked at the level of Bid cleavage, and cytochrome C is not released from mitochondria. Cytochrome C is released from mitochondria when TRAIL is used in combination with wortmanin and to a lesser extent cycloheximide.

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Annual Summary Report for Award Number DAMD 17-99-1-9516

Introduction

Androgen- insensitive human prostate cancer does not respond to chemotherapy and is eventually fatal. Therefore, novel strategies to treat prostate cancer in humans are necessary. TRAIL (TNF-related apoptotic inducing ligand) shares significant amino acid similarity to tumor necrosis factor TNF. Like TNF, TRAIL is capable of inducing apoptosis in many cancer cell types via binding to its receptors DR4 and DR5 (death receptor 4 and 5). Unlike TNF, whose extreme toxicity to normal tissues makes it unusable for clinical therapies, TRAIL has little or no toxicity to normal cells. TRAIL has thus become a potential therapeutic agent in cancer therapy. In this study, the cytotoxic effect of TRAIL was tested *in vitro* on six different prostate cancer cell lines. TRAIL induced apoptosis in five of six prostate cancer cell lines. The molecular mechanism of TRAIL-induced apoptosis in prostate cancer was also examined.

Body

In the last year, I received scientific training from the laboratory of Dr. Kraft, division of medical oncology, and the laboratory of Dr. Rodman, division of cardiovascular pulmonary research at University of Colorado Health Science Center. Through a combination of personal instruction by my mentors and in journal clubs and seminars, I have become much more familiar with current understanding of prostate cancer biology and treatment. To date, I have completed task 1 and task 2 proposed in my statement of work.

I have determined that five of six prostate cancer cell lines undergo apoptosis when incubated with TRAIL. TRAIL protein was made in large amount by expressing recombinant human TRAIL in methyltrophic yeast *Pichia Pastoris* as a fusion protein. Cell viability curves for six prostate cancer cell lines using varying amounts of TRAIL protein were carried out by MTT assay. The prostate cancer cell lines ALVA 31, PC-3 and DU 145 were highly sensitive to cell death induced by TRAIL while the cell line TSU-Pr1 and JCA1 were moderately sensitive and the LNCaP cell line was resistant.

DNA fragmentation analysis confirmed that cell death induced by TRAIL is indeed apoptosis. Apoptosis downstream marker caspase 3 and PARP were cleaved as well. Thus, these experiments established that prostate cancer cells are sensitive *in vitro* to TRAIL induced apoptosis and strongly suggest that this protein can be used as a therapeutic agent.

To demonstrate whether there is a general protein synthesis dependent antiapoptotic mechanism in TRAIL resistant cell line LNCaP, protein synthesis inhibitor cycloheximide was used in combination with different concentration of TRAIL to treat LNCaP cells. Cycloheximide by itself did not induce apoptosis but did induce apoptosis when added in combination with TRAIL. Cycloheximide enhanced the apoptotic effect of TRAIL.

LNCaP cells had been determined above to be resistant to TRAIL induced apoptosis. An important difference between this line and the others tested was that protein kinase AKT is constitutively activated in LNCaP cells, but not the others. When an activator of AKT, PI3 kinase, was inhibited using either wortmanin or Ly 294002, the LNCaP cells became sensitive to TRAIL induced apoptosis. Neither wortmanin nor Ly 294002 alone cause any toxic effect. Both cycloheximide and PI3 kinase inhibitors can increase apoptosis when combined with TRAIL, suggesting that specific agents can be used to overcome this resistance.

To define the molecular mechanism of TRAIL- induced apoptosis, a series of Western and Northern blot experiments were done to compare the expression of various components of the TRAIL signaling pathway among the six prostate cancer cell lines. The first component in the pathway are the TRAIL receptors DR4 and DR5. DR4 and DR5 are ubiquitously expressed in all six cell lines. No correlation was found between the expressions of two death receptors and sensitivity to TRAIL. Expression of two decoy receptors of TRAIL DcR1 and DcR2, which compete for TRAIL binding, were also tested on six cell lines. No correlation was found between the expression of decoy receptors and sensitivity to TRAIL. The second component in the pathway is caspase 8

which is recruited along with death receptors to form death inducing signaling complex (DISC). The formation of DISC is believed to trigger the activation of caspase 8 which eventually cause apoptosis. Apoptosis inhibitor cflip , a critical protein that blocks activation of caspase 8 was found expressed ubiquitously in all six cell lines. There is no correlation between Flip levels and response to TRAIL. However, low expression of caspase 8 was found in TRAIL resistant cell line LNCaP, which may indicate that downstream mitochondria signaling pathway is critical.

The next set of experiments examined the possibility that there was another block to activation of the caspase cascade and downstream apoptogenic activity of mitochondria signaling pathway. I determined whether TRAIL induces caspase 8 cleavage and the release of cytochrome C from mitochondria in LNCaP cell lines. I found that caspase 8 is cleaved but cytochrome C is not released in LNCaP cells treated with TRAIL. However, when cycloheximide or wortmanin is administered with TRAIL, caspase 8 is cleaved, cytochrome C is released from mitochondria, and apoptosis occurred. This indicates that the block of cytochrome C release from mitochondria occurs downstream of caspase 8 activation. To more precisely determine the biochemical step at which this occurs a series of proapoptotic proteins of BCl2 family, including Bcl2, Bclxl, Bid, Bax and Bad, needs to be tested on these six different cell lines.

So far, only Bid has been tested. In LNCaP cells treated with TRAIL alone, Bid fail to be cleaved, but Bid is cleaved when TRAIL is added in combination with cycloheximide or wortmanin to LNCaP cells. Bid cleavage in these cells is consistent with cytochrome c release from mitochondria.

Although high constitutive expression of upstream signal AKT in LNCaP cells may contribute to resistance of LNCaP to TRAIL treatment, more recent preliminary data from studying mitochondria signaling pathway suggests that the block of TRAIL–Induced apoptosis may occur because the proapoptotic proteins expression and dimerization varies in different cell lines. More detailed study about mitochondria apoptosis signaling in TRAIL-induced apoptosis is currently on going.

Key Research Accomplishments

Five of six prostate cancer cell lines undergo apoptosis when incubated with TRAIL is determined.

Cell viability curves of six human prostate cancer cell lines to varying amount of TRAIL protein is completed.

Protein synthesis inhibitor cycloheximide enhance the killing of TRAIL.

PI3 kinase inhibitor wortmanin and Ly 294002 enhance the killing of TRAIL.

The mechanism by which the prostate cancer cell lines LNCaP is resistant to TRAIL-induced apoptosis is examined.

No correlation was found between the sensitivity of cells to TRAIL and the expression of TRAIL receptors DR4 and DR5 4, decoy receptors for TRAIL DcR1 and DcR2.

Flip is expressed constituitively in all six prostate cell lines, no correlation is found between the sensitivity of TRAIL and the expression of Flip.

Low Caspase 8 expression was found in LNCaP cells. TRAIL induced apoptotic signaling in resistant cell line LNCaP is blocked at the level of Bid cleavage.

Cytochrome C is not released from mitochondria in LNCaP cells, cytochrome C is released from mitochondria when TRAIL is used in combination with wortmanin and to a lesser extent cycloheximide.

Reportable Outcomes

Manuscript entitled "Elevated Akt activity protects the prostate cancer cell line LNCaP from TRAIL-induced apoptosis" is submitted to Journal of Biological Chemistry.

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Elevated Akt activity protects the prostate cancer cell line LNCaP from TRAIL-induced apoptosis

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Running Title: PI-3 kinase/Akt pathway blocks TRAIL-induced apoptosis and BID cleavage

SUMMARY

We find that the prostate cancer cell lines ALVA-31, PC-3 and DU 145 are highly sensitive to apoptosis induced by TNF-Related-Apoptosis-Inducing-Ligand (TRAIL), while the cell lines TSU-Pr1 and JCA-1 are moderately sensitive, and the LNCaP cell line is resistant. LNCaP cells lack active lipid phosphatase PTEN, a negative regulator of the PI-3 kinase/Akt pathway, and demonstrate a high constitutive Akt activity. Inhibition of PI-3 kinase using wortmannin suppressed constitutive Akt activity and sensitized LNCaP cells to TRAIL. Although treatment of LNCaP cells with TRAIL induced cleavage of the caspase 8 and XIAP proteins, processing of BID, mitochondrial release of cytochrome c, activation of caspases 7 and 9 and apoptosis did not occur unless TRAIL was combined with wortmannin or cycloheximide. Blocking cytochrome c release by Bcl-2 overexpression rendered LNCaP cells resistant to TRAIL plus wortmannin treatment, but did not affect caspase 8 or BID processing. This indicates that in these cells mitochondria are required for the propagation rather than the initiation of the apoptotic cascade. Infection of LNCaP cells with an adenovirus expressing a constitutively active Akt reversed the ability of wortmannin to potentiate TRAIL-induced BID cleavage. Thus, the PI-3 kinase-dependent blockage of TRAIL-induced apoptosis in LNCaP cells appears to be mediated by Akt through the inhibition of BID cleavage.

INTRODUCTION

TRAIL (TNF-Related-Apoptosis-Inducing-Ligand) (1) also known as Apo-2 ligand (2) is a pro-apoptotic cytokine that together with three related proteins, TNFα, CD95/FasL and TWEAK/Apo3L constitutes a family of ligands that transduce death signals through death domain containing receptors (3-5). TRAIL is a type II transmembrane protein which functions by binding to two closely related receptors, DR4 and DR5 (6). Both TRAIL and its receptors are ubiquitously expressed (7), suggesting the existence of mechanisms that protect normal tissues from TRAIL-induced apoptosis.

TRAIL is capable of inducing apoptosis in a wide variety of cancer cells in culture and in tumor implants in mice, including cancers of the colon, breast, lung, kidney, CNS, blood and skin (1, 6, 8-11). At the same time, unlike TNFα and Fas ligand whose use for cancer therapy has been hampered by their severe toxicity (12, 13), TRAIL has no toxic effects when systemically administered in rodents (10) and non-human primates (9). Although the majority of normal human cells tested so far appear to be TRAIL-resistant, recent experiments demonstrated that cultured human liver cells may be sensitive to TRAIL (14), suggesting that additional studies are required to investigate what determines resistance or sensitivity to this agent.

Despite the ubiquitous expression of TRAIL receptors, a significant proportion of cell lines originating from various

cancer types demonstrate either partial or complete resistance to the pro-apoptotic effects of TRAIL. These findings suggest either defects in apoptotic pathways or the presence of inhibitors of TRAIL-induced apoptosis. The later possibility appears to be more likely, since the resistance of many types of cancer cells to TRAIL can be reversed by treatment with protein synthesis inhibitors (15-19), or chemotherapeutic agents (9, 11). Some normal human cells can also be sensitized to TRAIL by the inhibition of protein synthesis (20). The elucidation of mechanisms that control sensitivity to TRAIL may lead to better understanding of death receptor-mediated signaling and help to develop TRAIL-based approaches to cancer treatment.

Activation of death receptors leads to the formation of the death-inducing signaling complex (DISC) (21) which includes the receptor itself and caspase 8 (22). The recruitment of caspase 8 to TRAIL receptors DR4 and DR5 is thought to be mediated by the adaptor protein FADD (23-25). The formation of the DISC triggers autoprocessing and activation of caspase 8 (22) that in turn results in the cleavage and activation of the effector caspases 3 or 7 (26, 27), leading to apoptosis. Activated caspase 8 may also cleave a pro-apoptotic protein BID whose cleavage product triggers cytochrome c release from mitochondria (28, 29). In some but not all cell types, the mitochondrial step may be required to amplify the apoptotic signal and fully activate caspase 8 (30). As the TRAIL-induced apoptotic signal is a

multistep process, inhibition of this cascade may occur at several stages. For example, at the ligand-receptor level TRAIL signaling could be inhibited by the overexpression of non-functional TRAIL receptors DcR1 or DcR2 that compete for ligand binding (31), or by proteins that induce rapid internalization of TRAIL receptors (similar to Fas inhibition the adenoviral protein E3) (32). At the DISC, the apoptotic pathway may be inhibited by cFLIP protein that is capable of blocking processing and activation of caspase 8 (33, 34). Downstream of DISC, IAP proteins may specifically inhibit the executor caspases 3 and 7 (35). In those cells that require mitochondria to stimulate apoptosis, the signal may be inhibited by Bcl-2/Bcl-X_L types of proteins that prevent the release of pro-apoptotic factors from the mitochondria (30).

In the present study, we tested the cytotoxic effects of TRAIL on six human prostate cancer cell lines, demonstrating variable responses, with some cell lines being extremely sensitive and others highly resistant. The highly resistant cell line LNCaP was further investigated to examine mechanisms that protect it from TRAIL-mediated apoptosis. We find that the TRAIL-induced death signal in LNCaP cells is negatively regulated by a high constitutive activity of protein kinase Akt. Furthermore, the anti-apoptotic block occurs downstream of caspase 8 activation at the level of BID protein cleavage. This study is the first demonstration that the PI-3 kinase/Akt pathway may interfere with an apoptotic signal by inhibiting processing of BID.

EXPERIMENTAL PROCEDURES

Antibodies. Antibodies were obtained from the following sources: anti-phospho-Akt (New England Biolabs, Beverly, MA); anti-cytochrome c and anti-BID (Zymed Laboratories, So. San Francisco, CA); anti-Akt and anti-XIAP (Transduction Laboratories, Lexington, KY); anti-HA1 tag (BAbCO, Richmond, CA); anti-caspase 8 (Upstate Biotechnology, Lake Placid, NY); anti-caspase 7 (PharMingen, San Diego, CA); anti-caspase 9 (Oncogene Research Products, Boston, MA); anti- $FLIP_L$ (Affinity BioReagents, Golden, CO); anti-FLIPγ/δ (Calbiochem San Diego, CA).

Cell culture. Prostate cancer cell lines LNCaP, PC-3, DU 145, TSU-Pr1, JCA-1 and ALVA-31 were passaged in RPMI 1640 with 10% FCS, 50 U/ml penicillin and 50 U/ml streptomycin. The sources for these cell lines, their characterization and use in our laboratories have been described previously (36). LNCaP cells overexpressing Bcl-2 (37) were kindly provided by Dr. R. Buttyan (Columbia Presbyterian Medical Center, New York, NY) and grown in media supplemented with 400 μg/ml of G418.

Expression of recombinant TRAIL in yeast Pichia pastoris. A cDNA encoding for soluble human TRAIL (residues 114-281) was amplified by PCR from the EST clone 117926 (GeneBank accession number T90422) in-frame with N-terminal hexahistidine tag using oligonucleotides 5'-AGTCATGAATTCCATCACCATCACCATCACGTGAGAGAAAGAGGTCCTCAGAGAGTAG-3'

and 5'-AGTCATGGTACCTTAGCCAACTAAAAAGGCCCCGAAAAA-3'. This cDNA was then cloned into the EcoRI/KpnI sites of pPICZαA vector (Invitrogen, Carlsbad, CA) in-frame with the cleavable secretion signal from yeast α factor. All manipulations of yeast were performed in general as outlined in the Invitrogen manual. Briefly, the expression vector was linearized and transformed by electroporation into *Pichia pastoris* strain SMD1168 (38). Transformants were selected on 500 μg/ml of Zeocin and secretion of TRAIL tested by Western blotting. For large-scale production yeast were grown for 24 h in 10 L of BMGY buffered with 100 mM potassium phosphate buffer, pH 6.0 medium containing 0.2 ml of antifoam 289 (Sigma, Saint Louis, MO) at constant aeration and mixing to OD_{600} 15. To induce TRAIL production, cells were pelleted by centrifugation, resuspended in BMMY medium containing 0.5% methanol and grown for 24 h. The supernatant was concentrated using tangential flow Prep/Scale-TFF cartridge (Millipore, So. San Francisco, CA) and recombinant TRAIL purified by Ni-chelate chromatography on a Ni-NTA agarose column (QIAGEN, Valencia, CA). This procedure yielded about 2 mg of pure protein from 1 liter of yeast supernatant.

Cytotoxicity assays. Cell viability was determined spectrophotometrically using an MTS tetrazolium based assay (Promega, Madison, WI). Absorbance was measured at 490 nm and data from duplicate determinations was plotted as percent of untreated control cells. Quantitative analysis of DNA

fragmentation was done using a Cell Death Detection ELISA^{plus} kit (Roche Diagnostics Corporation, Indianapolis, IN) by measuring relative amounts of DNA-histone complexes released into the cytoplasm. Data from triplicate determinations were plotted as percent control of untreated cells. TUNEL assay was performed using the FragEL™ DNA fragmentation detection kit (Oncogene Research Products, Cambridge, MA).

Measurement of cytochrome c release from mitochondria. Cytosolic extracts from LNCaP cells were prepared by the hypotonic lysis procedure originally described by Bossy-Wetzel *et al.*, (1998) (39) and modified by Carson *et al.*, (1999) (40). LNCaP cells grown on 15-cm plates to 50% confluence were placed on ice and then scraped directly into growth media and centrifuged for 2 min at 200 X g. Cell pellets were then washed once with ice-cold PBS and resuspended in 300 µl of hypotonic lysis buffer (220 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH (pH 7.4), 50 mM KCl, 5 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT) containing protease inhibitors, including Complete Cocktail (Boehringer Mannheim, Germany), 1 mM PMSF, 10 µg/ml leupeptin, and 2 µg/ml aprotinin. Cells were incubated on ice for 45 min and homogenized by pipeting (10 passes up and down). Supernatants were cleared by 10 min centrifugation at 1000 x g, followed by 30 min at 100,000 x g and analyzed by Western blotting with the anti-cytochrome c antibody.

Construction of adenoviral vectors expressing myr-Akt. Full length coding sequence of human Akt1 was fused in frame with the myristoylation signal from human Src gene in the N-terminus, and HA-tag in the C-terminus (myr-Akt). Kinase dead construct was created by mutating lysine 179 for alanine, destroying in that way an ATP-binding site (myr-Akt (K-)). Recombinant adenoviruses were constructed by the method described by Crouzet *et al.*. (1997) (41). Briefly, cDNAs of interest were subcloned into the expression cassette in plasmid vector pXL2996 under the control of the CMV promoter. Each expression cassette was subcloned into the shuttle vector pXL3474. The resulting shuttle plasmids were introduced into *E. coli* JM83 cells by electroporation. After double homologous recombinations, plasmid DNA for recombinant virus was purified by CsCl density gradient centrifugation. This DNA was linearized and transfected into 293 cells. Two to three weeks after transfections, recombinant adenovirus was harvested from the conditioned media and amplified in 293 cells. The concentration of recombinant adenovirus stocks was determined by cytoplasmic toxicity assay.

RESULTS

Effect of soluble TRAIL on 6 prostate cancer cell lines. Recombinant human TRAIL (residues 114-281) was produced in methylotrophic yeast *Pichia pastoris* as a fusion protein containing N-terminal hexahistidine tag and a cleavable secretion signal from yeast α factor. These features allowed quick one-step purification of secreted 20 kDa TRAIL by Ni-chelate chromatography from yeast supernatant yielding approximately 2 mg of pure protein from each liter of yeast culture media (Fig. 1A). The cytotoxic effects of TRAIL were tested on a panel of six prostate cancer cell lines (Fig. 1B). Cell viability assays demonstrated that three of these cell lines, ALVA-31, DU 145 and PC-3 were very sensitive to TRAIL, JCA-1 and TSU-Pr1 revealed moderate sensitivity whereas LNCaP cells were resistant to as high as 4 μg/ml of TRAIL. Internuclosomal fragmentation (DNA laddering) confirmed that cell death occurred by apoptosis (data not shown).

Resistance of LNCaP cells to TRAIL correlates with elevated level of Akt/Protein kinase B activity. To investigate the mechanisms controlling the resistance of LNCaP cells to the cytotoxic effect of TRAIL, a series of Western and Northern blot experiments were done to compare the expression of various components of the TRAIL signaling pathway among the six prostate cancer cell lines. However, no correlation was found between the sensitivity of cells to

TRAIL and the expression of TRAIL receptors DR4 and DR5, decoy receptors for TRAIL DcR1 and DcR2, initiator caspase 8, and apoptosis inhibitory protein cFLIP (data not shown). LNCaP cells contain a deactivating frameshift mutation in the gene encoding the tumor suppresser PTEN (42). This dual specificity phosphatase cleaves D3 phosphate of second messenger lipid phosphatidylinositol (3,4,5) triphosphate (PIP-3) (43). PIP-3 produced by PI3-kinase activates protein kinase Akt and, therefore, the lack of negative regulation by PTEN results in the constitutive activation of Akt in LNCaP cells (40). Immunoblot analysis with an antibody that specifically recognizes the phosphorylated/activated form of Akt demonstrates that LNCaP cells possess the highest Akt activity among the six prostate cancer cell lines (Fig. 2A). Treating cells with the inhibitor of PI-3 kinase, wortmannin (200 nM) for 6 hours reverses the high constitutive activity of Akt (Fig. 2B).

Inhibition of PI-3 kinase activity or protein synthesis renders LNCaP cells sensitive to TRAIL. To test whether the high constitutive activity of Akt in LNCaP cells results in their resistance to TRAIL, we first examined how PI-3 kinase inhibitors wortmannin (200 nM) (Fig. 3) or LY294002 (20 μM) (data not shown) effect TRAIL cytotoxicity. Although both substances significantly enhanced the pro-apoptotic effects of TRAIL, LY294002 revealed significant toxicity and was not used in further studies. Apoptotic DNA fragmentation was quantitated by measuring the relative amounts of DNA-histone

complexes released into the cytoplasm. Figure 3A demonstrates that treatment of LNCaP cells with TRAIL (1 μg/ml) in combination with wortmannin (200 nM) resulted in greater DNA fragmentation than that caused by 100 μM of the potassium ionophore valinomycin, a potent inducer of apoptosis (44). A longer treatment (24 h) with TRAIL plus wortmannin resulted in the death of up to 100% of cells as judged by apoptotic morphology (Fig. 3B). Neither TRAIL nor wortmannin alone revealed any significant cytotoxicity. Treatment of LNCaP cells with cycloheximide in combination with TRAIL, but not each agent alone, induced apoptosis in LNCaP cells, albeit to a lesser extent than TRAIL plus wortmannin treatment. Thus, the resistance of LNCaP cells to TRAIL results from the blockage of the TRAIL-induced apoptotic signal transduction cascade rather than the defects in apoptotic machinery. This data demonstrates that the blockage of TRAIL-mediated apoptosis in LNCaP cells requires PI-3 kinase activity and involves some short-lived protein component(s).

TRAIL-mediated cytochrome c release is blocked in LNCaP cells. Depending on the cell type, apoptotic signaling mediated by CD95/Fas may or may not require the release of pro-apoptotic factors (cytochrome c and apoptosis-inducing factor) from mitochondria. In type II, but not in type I cells, inhibition of mitochondrial apoptogenic activities by overexpression of Bcl-2 protein blocks Fas-mediated apoptosis (30). To examine whether the apoptogenic activity of

mitochondria is required for the transduction of the TRAIL-induced death signal in LNCaP cells the cytotoxic effects of TRAIL alone or in combination with wortmannin were studied in a LNCaP cell line overexpressing Bcl-2 (37). Quantitation of apoptotic nuclei by TUNEL technique clearly demonstrates that Bcl-2 overexpression impairs the cytotoxic effect of TRAIL (Fig. 4A), indicating that mitochondria play an important role in TRAIL-induced apoptosis of LNCaP cells. If the resistance of LNCaP cells to TRAIL results from the high constitutive activity of Akt, this enzyme may block apoptosis either upstream (45, 46) or downstream (47) of mitochondrial cytochrome c release. To discriminate between these two possibilities, experiments were done to examine whether TRAIL-induced cytochrome c release is inhibited in LNCaP cells. LNCaP cells were incubated for six hours with TRAIL alone or TRAIL in combination with cycloheximide or wortmannin. Cytosolic extracts were then prepared under conditions that keep mitochondria intact (39), and cytochrome c released to the cytosolic fraction was then detected by immunoblotting. This experiment demonstrated that in LNCaP cells TRAIL alone does not trigger the release of cytochrome c from the mitochondria, but it does so in combination with wortmannin and, to a lesser extent, cycloheximide (Fig. 4B). Thus, TRAIL-induced apoptotic signaling in LNCaP cells is blocked upstream of the mitochondria.

TRAIL-induced apoptotic signaling in LNCaP cells is blocked at the level of BID cleavage. To understand at what

biochemical step the TRAIL-mediated apoptotic cascade is blocked in LNCaP cells, a series of immunoblotting experiments were carried out using antibodies to proteins involved in this cascade. Our results demonstrate that processing of initiator caspase 8 is induced by TRAIL alone as efficiently as when TRAIL is combined with cycloheximide and wortmannin (Fig. 5a). Similarly, these two compounds did not enhance TRAIL-induced cleavage of the apoptosis inhibitory protein XIAP, a substrate for several caspases including caspase 8 (48). These results suggest that the anti-apoptotic block in LNCaP occurs downstream of caspase 8 activation. In contrast, proteolytic cleavage of the caspase 8 substrate BID was not detected in TRAIL-treated cells unless TRAIL was administered in combination with cycloheximide or wortmannin. Caspase 8-mediated cleavage of BID generates a proteolytic fragment, tBID that is capable of inducing mitochondrial cytochrome c release and providing a functional link between death receptors and the mitochondria (28,29). The lack of BID cleavage is thus consistent with the observation that TRAIL alone is not capable of inducing cytochrome c release. TRAIL-mediated processing of cytochrome c-dependent caspase 9 and effector caspase 7 were also detected only if TRAIL was combined with wortmannin or cycloheximide. Thus, the PI-3 kinase- and protein synthesis-dependent anti-apoptotic block in LNCaP cells occurs downstream of caspase 8, at the level of BID cleavage.

Alternatively, it is possible that the lack of BID cleavage may result from an inhibition of mitochondrial function. By analogy with the CD95/Fas system, LNCaP cells may be classified as type II cells since mitochrondrial function appears to be necessary for apoptosis. In type II cells mitochondrial cytochrome c release serves as an amplification loop that potentiates the activation of caspase 8. If a similar mitochondria-dependent amplification loop is involved in TRAIL signaling in LNCaP cells, its disruption may affect caspase 8-mediated BID cleavage. To test whether or not cleavage of BID in LNCaP cells depends on mitochondrial function, the processing of BID in Bcl-2 overexpressor LNCaP cells versus parental cells was examined. Immunoblot analysis (Fig. 5B) demonstrates that after six hours of treatment with TRAIL plus wortmannin or TRAIL plus cycloheximide BID is processed equally well in parental and Bcl-2 overexpressing LNCaP cells. In addition, caspase 8 was processed efficiently (Fig. 5a) in both cell lines as judged by the TRAIL-induced appearance of a cleavage product which corresponds to the 20-kDa active subunit of caspase 8. Thus, apoptogenic activity of mitochondria is not required for TRAIL-induced cleavage of BID and caspase 8.

Our results demonstrate that the blockage of TRAIL-induced apoptosis at the level of BID cleavage can be removed by cycloheximide treatment, suggesting the possibility that this inhibition may be mediated by a short-lived protein. It has been hypothesized that inhibition of protein synthesis

sensitizes cells to death-inducing ligands by down-regulating anti-apoptotic cFLIP proteins (15, 19, 49). To determine whether this is the case for LNCaP cells, cell lysates from a previous experiment (Fig. 5A) were immunoblotted with antibodies that recognize different splice variants of cFLIP proteins: $FLIP_L$, FLIPγ and FLIPδ (50). In contrast to published data, treatment of LNCaP cells for up to sixteen hours with cycloheximide or wortmannin had no effect on the level of cFLIP proteins (Fig. 5C), suggesting that they are unlikely to be involved in the inhibition of TRAIL signaling in LNCaP cells.

Constitutively active Akt blocks TRAIL/wortmannin-induced BID cleavage. The potentiating effect of wortmannin on TRAIL-induced BID cleavage suggests that Akt may be involved in the inhibition of TRAIL signaling in LNCaP cells. To confirm this hypothesis, a constitutively active Akt, constructed by fusing Akt to the myristoylation signal of Src protein (myr-Akt) was introduced into LNCaP cells by adenovirus-mediated gene transfer. If Akt is the sole target of the wortmannin effect, then this infection would be expected to counteract the ability of wortmannin to sensitize LNCaP cells to TRAIL-induced BID cleavage. As a control, an adenovirus containing kinase-inactive Akt (myr-Akt(K-) was used. LNCaP cells infected with adenoviral constructs sixteen hours prior to the experiment were treated for additional six hours with TRAIL or TRAIL plus wortmannin and BID cleavage was examined by immunoblotting. Our results demonstrate (Fig.

6) that the infection of LNCaP cells with myr-Akt, but not with the kinase-inactive Akt, inhibits processing of BID induced by TRAIL plus wortmannin treatment. TRAIL-mediated cell death was also inhibited in myr-Akt infected cells as judged by cell morphology (data not shown). Thus, activated Akt is capable of rescuing LNCaP cells from the apoptogenic action of TRAIL plus wortmannin treatment, supporting the hypothesis that the resistance of LNCaP cells to TRAIL results from high constitutive activity of Akt.

DISCUSSION

We have developed a novel approach to obtaining preparative amounts of pro-apoptotic ligand TRAIL and tested the effects of this reagent on a panel of six prostate cancer cell lines. Soluble TRAIL was produced by a methylotrophic yeast *Pichia pastoris*, secreted into the media, and then purified to homogeneity by one-step chromatography on a Ni-chelate column. Cytotoxicity assays demonstrated that 3 cell lines, ALVA-31, DU 145, and PC-3, were very sensitive to TRAIL, while in comparison JCA-1 and TSU-Pr1 revealed moderate sensitivity, and LNCaP cells were resistant to as high as 4 μg/ml of TRAIL. Comparing these results with the data published on Fas ligand-induced apoptosis indicates that prostate cancer cells differ in their responses to these two apoptotic stimuli. Whereas cells believed to be derived from primary prostate cancer tumors (ALVA-31, JCA-1), were reported to be sensitive to Fas ligand-induced apoptosis, cells originating from distant metastasis (DU 145, PC-3, TSU-Pr1 and LNCaP) appeared to be Fas-resistant despite the expression of Fas antigen on the cell surface (36, 51). In contrast, among the above listed cell lines, only LNCaP cells were resistant to TRAIL-induced apoptosis, indicating that TRAIL has a greater potential as an agent to treat metastatic prostate cancer. These data also suggest that despite the similarity of CD95/Fas and TRAIL receptors, TRAIL and Fas ligand-mediated apoptosis may employ different signal

transduction pathways or be negatively regulated by different mechanisms in these prostate cancer cells.

We found that among six prostate cancer cell lines examined, the LNCaP cells which are the most highly resistant to TRAIL-induced apoptosis, have the highest constitutive activity of the Akt protein kinase. This result is consistent with the lack of the functional tumor suppressor PTEN, a negative regulator of the PI-3 kinase/Akt pathway in these cells (42). Because the Akt protein kinase is known to block apoptosis (52) we tested whether inhibition of this pathway affects the sensitivity of LNCaP cells to TRAIL. We found that treatment with the PI-3 kinase inhibitor wortmannin or the protein synthesis inhibitor cycloheximide renders them sensitive to TRAIL-induced apoptosis. Thus, the resistance of LNCaP cells to TRAIL results not from defects in apoptotic machinery, but from PI-3 kinase-dependent inhibition of the TRAIL-mediated apoptotic signaling pathway.

It has been reported that apoptosis induced by triggering of CD95/Fas (53, 54) is counteracted by the PI-3 kinase/Akt pathway, but the molecular mechanisms that cause apoptosis resistance remain unclear. To identify which step of the TRAIL-mediated apoptotic pathway is blocked in LNCaP cells, we first tested whether the release of pro-apoptotic factors from mitochondria is essential for TRAIL-induced death of these cells. The involvement of mitochondria in apoptosis induced by death receptors remains controversial. Scaffidi *et al.* (1998) (30) has proposed that 2 types of

cells exist that differ with respect to their requirement for mitochondria during Fas-mediated apoptosis. In type I cells caspase 8 is activated without involvement of mitochondria to a level sufficient to process the effector caspase 3. In contrast, in type II cells a mitochondria-dependent amplification loop is required to fully activate caspase 8 and transduce an apoptotic signal. This model has recently been questioned by Huang *et al.* (1999) who argues that the difference between type I and type II cells is an artifact of using agonistic ani-Fas antibodies to trigger Fas signaling instead of Fas ligand (55). To clarify the role of mitochondria in TRAIL induced apoptosis in LNCaP cells, we used Bcl-2 overexpressing LNCaP cells, which were shown to exhibit an impaired cytochrome c release in response to various apoptotic stimuli (37). Our results demonstrate that these cells are much more resistant to TRAIL plus wortmannin induced apoptosis compared to the parental cells. In these experiments, apoptosis was triggered by soluble death receptor ligand and not agonistic antibody, supporting the notion that in some cells mitochondrial function is indeed essential for death receptor-mediated apoptosis.

Using a cell fractionation approach we have found that TRAIL-induced cytochrome c release was blocked in LNCaP cells, but both wortmannin and cycloheximide are capable of overcoming this block. Release of mitochondrial cytochrome c by death receptors is triggered by a multistep mechanism. The formation of the DISC results in autoprocessing and

activation of the initiator caspase 8 followed by cleavage of the pro-apoptotic protein BID (28,29). A proteolytic fragment of BID translocates to the mitochondria as an integral membrane protein and triggers the release of mitochondrial cytochrome c (56). Using immunoblot analysis we found that cleavage of caspase 8 and one of its substrates, the anti-apoptotic protein XIAP (48) were induced by TRAIL alone as efficiently as when TRAIL was combined with either wortmannin or cycloheximide. This important result indicates that DISC formation or caspase 8 activation was not blocked in LNCaP cells. In contrast, wortmannin and cycloheximide were required for TRAIL-induced cleavage of BID, the release of cytochrome c, as well as processing of caspases 9 and 7. Thus, the PI-3 kinase dependent block of TRAIL-induced apoptosis in LNCaP cells occurs at the level of BID cleavage.

The requirement for mitochondrial apoptogenic activity in TRAIL-induced death suggests that LNCaP cells are similar to type II cells. If so, the lack of BID cleavage could, in principle, be explained by the disruption of a mitochondria-dependent amplification loop resulting in only partial activation of caspase 8. To see whether this hypothesis could be true, we compared the cleavage of BID and caspase 8 in Bcl-2 overexpressing versus parental LNCaP cells and found that these proteins are processed equally well in both cell lines. These results demonstrate that although mitochondrial function is important for TRAIL-induced apoptosis in LNCaP cells, unlike "typical" type II cells mitochondria are

required not to amplify caspase 8 activation but to transduce apoptotic signal downstream of the initiator caspase. Therefore, it may be possible to classify LNCaP as type III cells where mitochondria are involved in the propagation rather than the initiation of the apoptotic cascade.

Involvement of PI-3 kinase in the block of apoptosis suggests that Akt could mediate resistance of LNCaP cells to TRAIL. To confirm this hypothesis, we tested whether overexpression of constitutively active Akt could inhibit the pro-apoptotic effect of TRAIL plus wortmannin treatment. For this purpose we used a myristoylated derivative of Akt which exhibits kinase activity independently of PI-3 kinase (57). Both apoptosis (data not shown) and BID cleavage induced by treatment of LNCaP cells with TRAIL plus wortmannin were inhibited by overexpression of myristoylated Akt, indicating that resistance of LNCaP cells to TRAIL is, at least in part, mediated by Akt.

It has been documented that Akt may inhibit a variety of apoptotic stimuli in multiple ways (52). These include direct phosphorylation and modulation of pro-apoptotic proteins BAD (45) and caspase 9 (47), activation of anti-apoptotic NFκB-mediated transcriptional pathways (58, 59), or phosphorylation of the Forkhead family of transcription factors preventing them from inducing the transcription of pro-apoptotic genes (60). Inhibition of BID cleavage has not been previously reported as a mechanism through which PI-3 kinase and Akt block apoptotic signals.

Although it remains unclear how the PI-3 kinase/Akt pathway mediates inhibition of BID cleavage, our data suggest an indirect mechanism. First, inhibition of protein synthesis by cycloheximide affected the same step of TRAIL apoptotic cascade as the inhibition of PI-3 kinase, suggesting that a short-lived protein is involved in the PI-3 kinase mediated blockage. Second, the effect of myristoylated Akt appears to be cell type specific since its overexpression in HeLa or ALVA-31 cells did not rescue these cells from TRAIL-induced apoptosis or BID cleavage (data not shown).

It has been reported that short-term (three to seven hours) treatment of human keratinocytes (49), HeLa and Kym-1 cells (19) with cycloheximide significantly reduces the level of cellular cFLIP protein. Since upon overexpression cFLIP is capable of inhibiting Fas-mediated apoptosis (33, 34), it has been suggested that protein synthesis inhibitors sensitize cells to TRAIL by down-regulating cFLIP. To examine this hypothesis, we tested the level of various splice variants of cFLIP ($FLIP_L$, FLIPγ and FLIPδ) in LNCaP cells and found that neither cycloheximide nor wortmannin treatment affected cFLIP levels after as long as sixteen hours of treatment. These data are consistent with our observation on renal carcinoma cells (61) and published results on Kaposi's sarcoma cells (17) in which that inhibition of protein synthesis sensitized cells to TRAIL without affecting the expression of cFLIP proteins. Thus, mediators of the PI-3 kinase-dependent

blockage of TRAIL-induced BID cleavage and apoptosis in LNCaP cells still await identification and characterization.

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FIGURE LEGENDS

Fig. 1. Sensitivity of human prostate cancer cell lines to soluble human TRAIL. A, purification of recombinant TRAIL from *Pichia Pastoris* supernatant by Ni-chelate chromatography. B, relative viability of six prostate cancer cell lines treated for 24 h with TRAIL, as measured by the MTS assay. Data are expressed as the means for duplicate determinations.

Fig. 2. Constitutive activity of Akt in prostate cancer cells determined by immunoblot with anti-Phospho-Akt antibody (Ser473). A, high constitutive activity of Akt in LNCaP cells when compared to other prostate cancer cell lines. Cell lysates prepared from six prostate cancer cell lines were probed by immunoblotting with anti-Phospho-Akt antibody (upper panel) or anti-Akt antibody (bottom panel). B, inhibition of constitutive Akt phosphorylation in LNCaP cells by wortmannin. LNCaP cells were treated with wortmannin (200 nM) or cycloheximide (10 μM) for 6 hours and cell lysates were immunoblotted with anti-Phospho-Akt antibody (upper panel) or anti-Akt/PKBα antibody (bottom panel).

Fig.3. Wortmannin and cycloheximide potentiate the cytotoxic activity of TRAIL. A, LNCaP cells were treated

for 6 h with 1 μg/ml of TRAIL, 200 nm of wortmannin (WM), or 10 μM of cycloheximide (CHX) alone or in combinations. DNA fragmentation was quantitated by measuring the relative amounts of DNA-histone complexes released into the cytoplasm using a Cell Death Detection ELISA kit. B, LNCaP cells were treated for 24 h as described above and visualized by light microscopy.

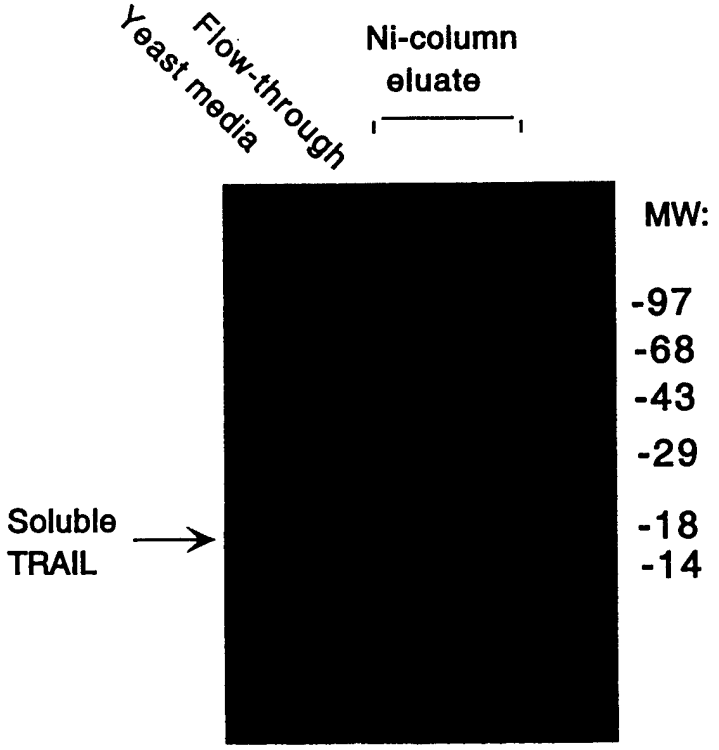
Fig. 4. The role of mitochondrial cytochrome c release for TRAIL-induced apoptosis in LNCaP cells. A, TRAIL-induced cytochrome c release is blocked in LNCaP cells. LNCaP cells were treated with TRAIL, wortmannin or cycloheximide as described in the legend to Figure 3A. Cells were lysed in hypotonic buffer and cytochrome c in the cytosolic fraction was measured by immunoblotting with cytochrome c-specific antibodies. B, apoptogenic function of mitochondria is essential for TRAIL-mediated apoptosis in LNCaP cells. Parental LNCaP cells or LNCaP cells overexpressing Bcl-2 were treated as described above and apoptotic nuclei were scored by TUNEL staining. Several randomly chosen microscopic fields were visualized and both normal and TUNEL - positive cells counted. The numbers of TUNEL-positive versus total numbes of counted cells are represented as ratios above bar graphs.

Fig. 5. Block of TRAIL- mediated apoptotic signal in LNCaP cells occurs at the level of BID cleavage. A,

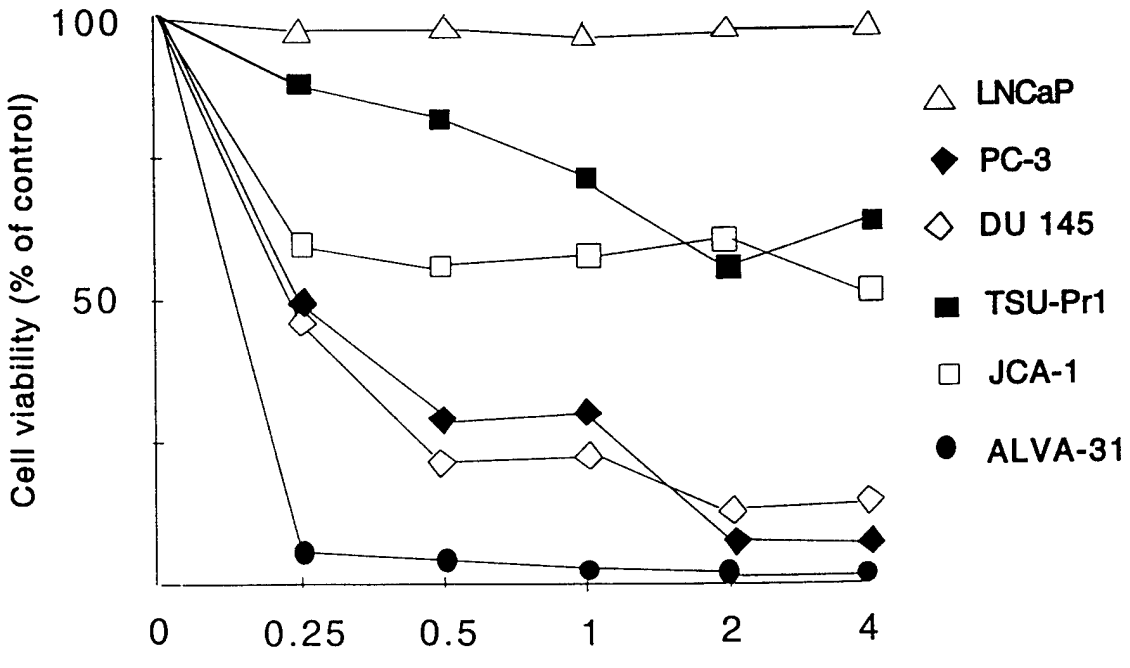
wortmannin and cycloheximide potentiate TRAIL- induced cleavage of BID, caspase 9 and caspase 7, but do not affect processing of caspase 8 and XIAP. LNCaP cells were treated for 6 or 16 h with 1 μg/ml of TRAIL, 200 nm of wortmannin (WM), or 10 μM of cycloheximide (CHX) alone or in combinations. Cell lysates were electrophoresed and consecutively immunoblotted with antibodies specific to caspase 8, XIAP, BID, caspase 9 and caspase 7. Arrows on the left indicate cleavage products. B, apoptogenic activity of mitochondria is not required for TRAIL-induced cleavage of BID and caspase 8. Parental LNCaP cells and LNCaP cells overexpressing Bcl-2 were treated for 6 h with 1 μg/ml of TRAIL and 200 nM of wortmannin alone or in combination. Cleavage of caspase 8 and BID was analyzed by immunoblotting with the corresponding antibodies. Blots were processed by ECL and two different exposures were taken to visualize holo caspase 8 (short exposure) and its 20 - kDa proteolytic fragment (long exposure). Arrow indicates caspase 8 cleavage product. C, TRAIL sensitivity does not correlate with cFLIP levels in LNCaP cells. Cell lysates from the experiment described in the Figure 5A legend were immunoblotted with antibodies that specifically recognize different splice variants of cFLIP protein: $FLIP_L$, FLIPγ and FLIPδ.

Fig.6. Constitutively active Akt protects BID from cleavage induced by TRAIL plus wortmannin treatment. LNCaP cells were infected with adenoviral constructs expressing myristoylated Akt (myr-Akt) or kinase inactive myristoylated Akt (myr-Akt(K-)). Control cells were not infected with adenovirus. 16 h post-infection the cells were treated for 6 h with 1 μg/ml of TRAIL and 200 nM of wortmannin alone or in combination. Cell lysates were consecutively probed with BID-specific antibody and anti-HA1 antibody that recognizes hemagglutinin-tagged myr-Akt.

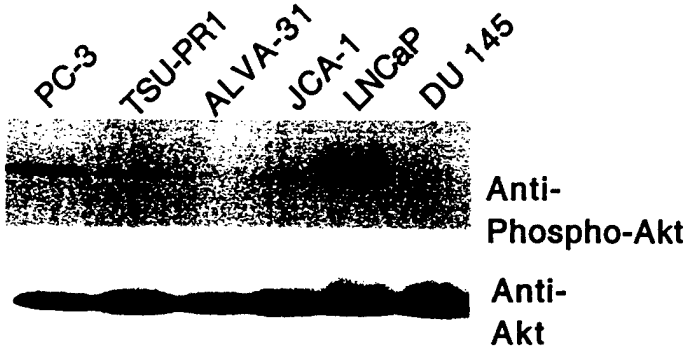
A


Yeast media
Flow-through
Ni-column eluate
MW:
-97
-68
-43
-29
-18
-14
Soluble TRAIL

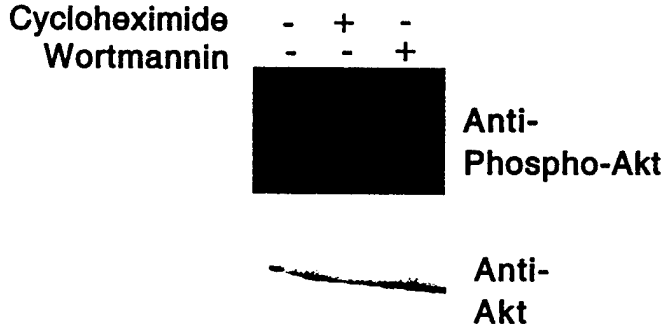
B


100
50
Cell viability (% of control)
0
0.25
0.5
1
2
4
LNCaP
PC-3
DU 145
TSU-Pr1
JCA-1
ALVA-31

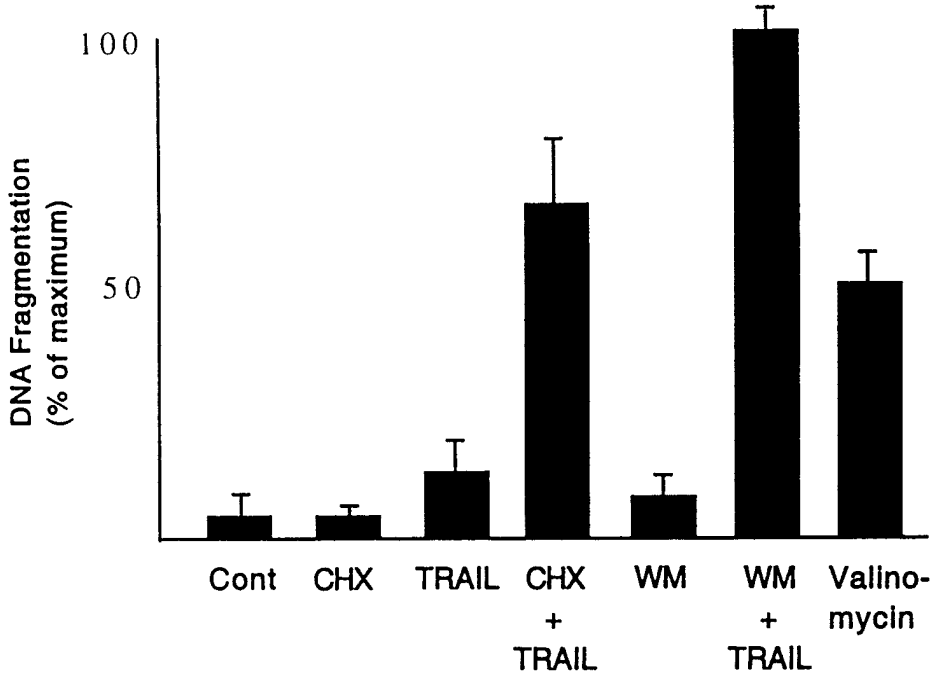
A


PC-3
TSU-PR1
ALVA-31
JCA-1
LNCaP
DU 145
Anti-
Phospho-Akt
Anti-
Akt

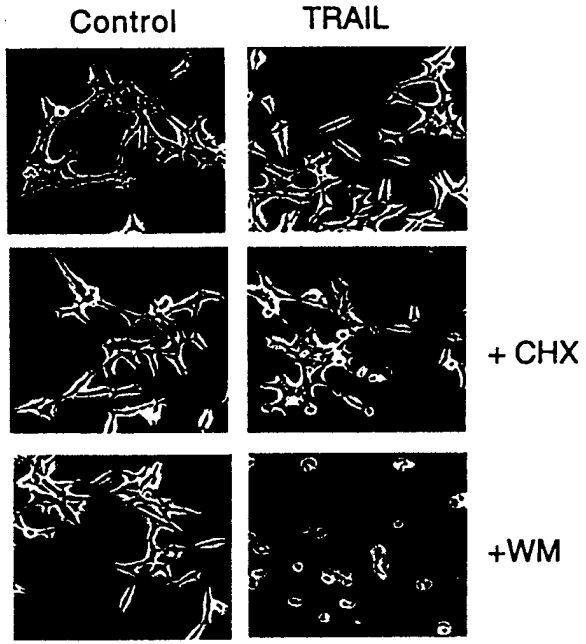
B

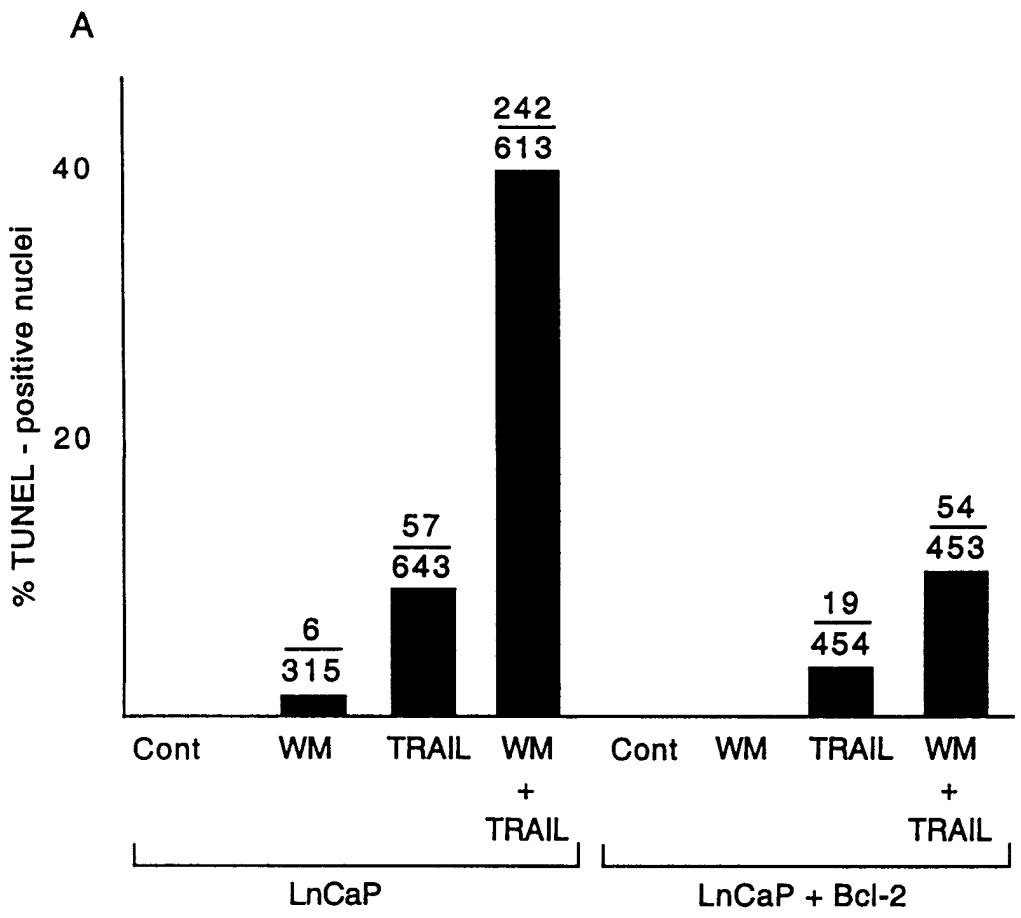

Cycloheximide - + -
Wortmannin - - +
Anti-
Phospho-Akt
Anti-
Akt

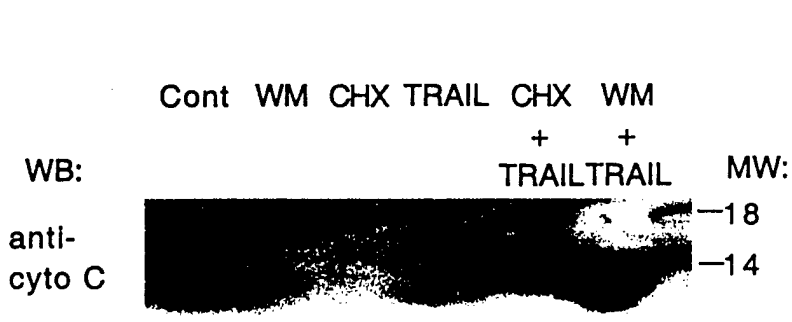
A

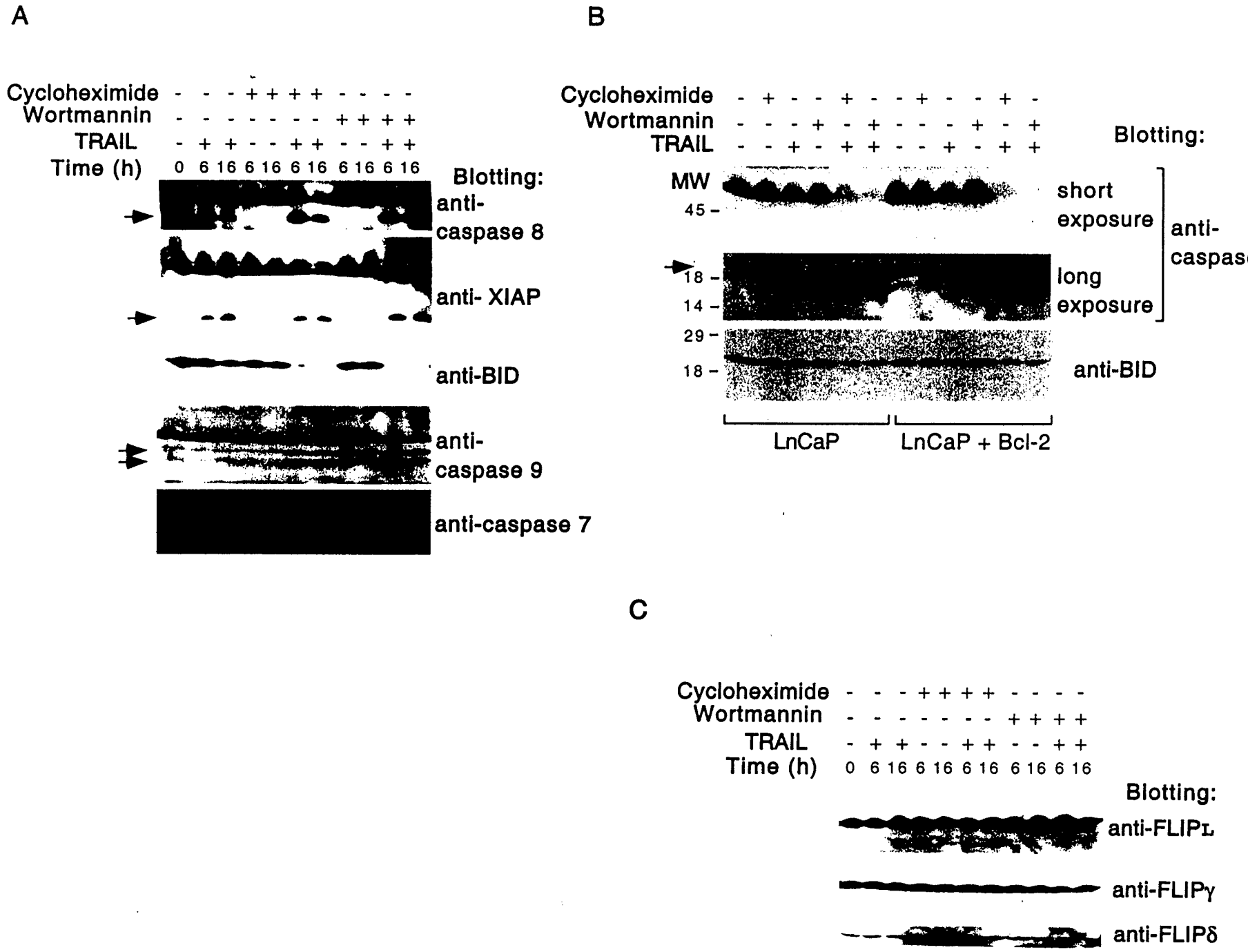

100
50
DNA Fragmentation
(% of maximum)
Cont
CHX
TRAIL
CHX
+
TRAIL
WM
WM
+
TRAIL
Valino-
mycin

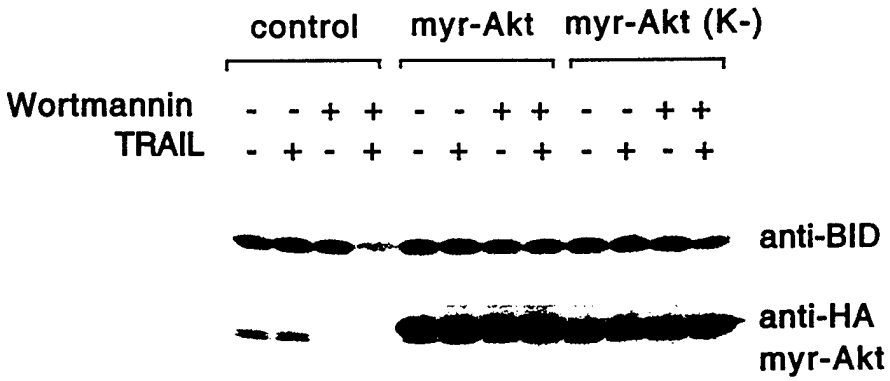
B


Control
TRAIL
+ CHX
+WM


A
% TUNEL - positive nuclei
40
20
242/613
57/643
6/315
19/454
54/453
Cont
WM
TRAIL
WM + TRAIL
Cont
WM
TRAIL
WM + TRAIL
LnCaP
LnCaP + Bcl-2


B
Cont
WM
CHX
TRAIL
CHX + TRAIL
WM + TRAIL
WB:
MW:
anti-cyto C
18
14


A
Cycloheximide - - - + + + + - - - -
Wortmannin - - - - - - - + + + +
TRAIL - + + - - + + - - + +
Time (h) 0 6 16 6 16 6 16 6 16 6 16
Blotting:
anti-caspase 8
anti- XIAP
anti-BID
anti-caspase 9
anti-caspase 7
B
Cycloheximide - + - - + - - + - - + -
Wortmannin - - - + - + - - - + - +
TRAIL - - + - + + - - + - + +
Blotting:
MW
45 -
short exposure
anti-caspase
18 -
14 -
long exposure
29 -
18 -
anti-BID
LnCaP
LnCaP + Bcl-2
C
Cycloheximide - - - + + + + - - - -
Wortmannin - - - - - - - + + + +
TRAIL - + + - - + + - - + +
Time (h) 0 6 16 6 16 6 16 6 16 6 16
Blotting:
anti-FLIPL
anti-FLIPγ
anti-FLIPδ


control
myr-Akt
myr-Akt (K-)
Wortmannin
TRAIL
- - + + - - + + - - + +
- + - + - + - + - + - +
anti-BID
anti-HA
myr-Akt