Elevated Akt Activity Protects the Prostate Cancer Cell Line LNCaP from TRAIL-induced Apoptosis*

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We find that the prostate cancer cell lines ALVA-31, PC-3, and DU 145 are highly sensitive to apoptosis induced by TRAIL (tumor-necrosis factor-related apoptosis-inducing ligand), while the cell lines TSU-Prl 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 phosphatidylinositol (PI) 3-kinase/Akt pathway, and demonstrate a high constitutive Akt activity. Inhibition of PI 3-kinase using wortmannin and LY-294002 suppressed constitutive Akt activity and sensitized LNCaP cells to TRAIL. Treatment of LNCaP cells with TRAIL alone induced cleavage of the caspase 8 and XIAP proteins. However, processing of BID, mitochondrial release of cytochrome c, activation of caspases 7 and 9, and apoptosis did not occur unless TRAIL was combined with either wortmannin, LY-294002, or cycloheximide. Blocking cytochrome c release by Becl-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.

TRAIL (tumor-necrosis factor-related apoptosis-inducing ligand) (1) also known as Apo-2 ligand (2) is a proapoptotic cytokine that together with three related proteins (tumor necrosis factor-α, CD95L/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 that 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, central nervous system, blood, and skin (1, 6, 8–11). At the same time, unlike tumor necrosis factor-α 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 nonhuman primates (9). Although the majority of normal human cells tested so far appear to be TRAIL-resistant, recent experiments have 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 TRAIL-induced apoptosis. These findings suggest either defects in apoptotic pathways or the presence of inhibitors of TRAIL-induced apoptosis. The latter 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)1 (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 caspase 3 or 7 (26, 27), leading to apoptosis. Activated caspase 8 may also cleave a proapoptotic 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). Since 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 nonfunctional TRAIL receptors DcR1 or DcR2 (31) or by proteins that induce rapid internalization of TRAIL receptors (32).
(similar to Fas inhibition the adenosinergic 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-XL types of proteins that prevent the release of proapoptotic 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 antiapoptotic 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 Inc.); anti-Akt and anti-XIAP (Transduction Laboratories, Lexington, KY); anti-HA1 tag (Babco, Richmond, CA); anti-caspase 8 (Upstate Biotechnology, Inc., Lake Placid, NY); anti-caspase 7 (PharMingen, San Diego, CA); anti-caspase 9 (Oncogene Research Products, Boston, MA); anti-FLIP, 7A (Affinity BioReagents, Golden, CO); anti-FLIP/β (Calbiochem).

**Cell Culture**—Prostate cancer cell lines LNCaP, PC-3, DU 145, TSU-Prl, JCA-1, and ALVA-31 were passaged in RPMI 1640 with 10% fetal calf serum (Invitrogen, Carlsbad, CA) in frame with the cleavable secretion signal from yeast α factor. All manipulations of yeast were performed under conditions described in the Invitrogen manual. Briefly, the expression vector was linearized and transformed by electroporation into P. pastoris strain SMD1168 (38). Transformants were selected on 50 μg/ml of zeocin, and secretion of TRAIL was tested by Western blotting. For large scale production, yeast were grown for 24 h in 10 liters of complex medium containing glycerol and antifoam 289 (Sigma, St. Louis, MO) and buffered with 100 mM potassium phosphate buffer, pH 6.0, at constant aeration and mixing to an A600 of 15. To induce TRAIL production, cells were pelleted by centrifugation, resuspended in complex medium containing 0.5% methanol, and grown for 24 h. The supernatant was concentrated using tangential flow PrepScale-TFF cartridge (Millipore Corp., Bedford, MA) and recombinant TRAIL purified by nickel-chelate chromatography on a Ni2+–nitrilotriacetic acid-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 a Cell Death Detection ELISA kit (Roche Diagnostics Corp., Indianapolis, IN) by measuring relative amounts of DNA-histone complexes released into the cytoplasm. Data from triplicate determinations were plotted as percentage of control of untreated cells. A TUNEL assay was performed using the FragEL DNA fragmentation detection kit (Onco-}

**PI 3-Kinase Blocks TRAIL-Induced Apoptosis and BID Cleavage**

**Results**

**Effect of Soluble TRAIL on Six Prostate Cancer Cell Lines**— Recombinant human TRAIL (residues 114–281) was produced in methylothrophic yeast P. pastoris as a fusion protein containing an 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 nickel-chelate chromatography from yeast supernatant yielding ~2 mg of pure protein from each liter of yeast culture medium (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-Prl revealed moderate sensitivity, whereas LNCaP cells were resistant to as high as 4 μg/ml of TRAIL. Internucleosomal fragmentation (DNA laddering) confirmed that cell death occurred by apoptosis (data not shown).

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 DcR1 and DcR2, initiator caspase 8, 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 suppressor PTEN (42). This dual specific phosphatase cleaves D3 phosphate of second messenger lipid phosphatidylinositol (PI) 3,4,5-trisphosphate (43). PI 3,4,5-trisphosphate produced by 3-kinase activation protein kinase Akt, and therefore, the lack of activity of PTEN resulted in constitutive activation of Akt in LNCaP cells (40). Immunoblot analysis with an antibody that specifically recognizes the phosphorylated/activated form of Akt (Ser172) demonstrates that LNCaP cells possess the highest Akt activity among the six prostate cancer cell lines (Fig. 2A). Treatment with the inhibitor of PI 3-kinase, wortmannin (200 nM), for 6 h
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) and LY-294002 (20 μM) effect TRAIL cytotoxicity. Wortmannin acts at nanomolar concentrations by covalently modifying PI 3-kinase (44) but is unstable in aqueous solutions (45), making it possible that some PI 3-kinase activity can be restored by de novo synthesis in the course of the experiment. LY-294002 does not bind the enzyme covalently and has an IC_{50} value for PI 3-kinase about 500-fold higher than that of wortmannin (46) but is much more stable in culture medium. We have found that both substances significantly enhanced the proapoptotic activity of TRAIL in LNCaP cells as judged by apoptotic morphology (Fig. 3A) and DNA fragmentation (Fig. 3B), quantitated by measuring the relative amounts of DNA-histone complexes released into cytoplasm. Since wortmannin and LY-294002 inhibit PI 3-kinase by different mechanisms, this result confirms that sensitization of cells to TRAIL occurs through the inhibition of the PI 3-kinase pathway. Inhibition of protein synthesis with cycloheximide also sensitized LNCaP cells to TRAIL (Fig. 3, A and B). The DNA fragmentation induced by TRAIL in combination with wortmannin, LY-294002, or cycloheximide was greater than that triggered by the potassium ionophore valinomycin (Fig. 3B), a potent inducer of apoptosis (47). 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. These data demonstrate that the inhibition 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 proapoptotic 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 an LNCaP cell line overexpressing Bcl-2 (37). Quantitation of apoptotic nuclei by the TUNEL technique clearly demonstrates that Bcl-2 overexpression impairs the cytoxic 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 (48, 49) or downstream (50) 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 6 h 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 (Fig. 4B). 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. 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 (51). These results suggest that the antiapoptotic 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. The involvement of PI 3-kinase in the blockage of TRAIL-induced BID cleavage was further confirmed by the experiment with another PI 3-kinase inhibitor, LY-294002. Fig. 5B demonstrates that treatment of LNCaP cells with LY-294002 in combination with TRAIL results in the decreasing of cellular BID level. Thus, the PI 3-kinase- and protein synthesis-dependent antiapoptotic 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 mitochondrial 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 Bel-2 overexpressor LNCaP cells versus parental cells was examined. Immunoblot analysis (Fig. 5C) demonstrates that after 6 h of treatment with TRAIL plus wortmannin or TRAIL plus cycloheximide, BID is processed equally well in parental and Bel-2-overexpressing LNCaP cells. In addition, caspase 8 was processed efficiently in both cell lines as judged by the TRAIL-induced appearance of a cleavage product that corresponds to the 20-kDa active subunit of caspase 8. Thus, apoptotic 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 antiapoptotic cFLIP proteins (15, 19, 52). 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: FLIPs, FLIPγ, and FLIPδ (53). In contrast to published data, treatment of LNCaP cells for up to 16 h 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 16 h prior to the experiment were treated for an additional 6 h with TRAIL or TRAIL plus wortmannin, and BID cleavage was examined by immunoblotting. Our results demonstrate (Fig. 6A) 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 apoptotic action of TRAIL plus wortmannin treatment, supporting the hypothesis that the resistance of LNCaP cells to TRAIL results from high constitutive activity of Akt.

We next tested whether activated Akt can also inhibit cleavage of BID induced by TRAIL plus cycloheximide treatment. However, no rescue was observed even when the adenovirus titer was 16 times higher than that sufficient to inhibit proapoptotic effects of TRAIL plus wortmannin treatment (Fig. 6B). These results suggest that the protective effects of Akt on BID cleavage may require Akt-induced protein synthesis.

Our results (Figs. 1B and 2A) indicate the existence of TRAIL-sensitive cell lines that possess an elevated Akt activity, albeit at a much lower level than that found in LNCaP cells. This result raises the question of whether the protective effect of Akt is cell type-specific or it occurs only when the level of Akt activity is above a certain threshold. To examine these possibilities, we overexpressed myristoylated Akt in various TRAIL-sensitive cell lines: DU 145 and ALVA-31 prostate cancer cells, A498 renal cancer cells, and HeLa cervical cancer cells. Of them, only ALVA-31 cells acquired significant resistance to TRAIL upon myr-Akt overexpression (Fig. 6C). Thus, the protective effect of Akt appears to be cell type-specific.

DISCUSSION

We have developed a novel approach to obtaining preparative amounts of proapoptotic 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 *P. pastoris*, secreted into the medium, and then purified to homogeneity by one-step chromatography on a nickel-chelate column. Cytotoxicity assays demonstrated that three 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 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 and 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, 54). 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

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Fig. 5. Block of TRAIL-mediated apoptotic signal in LNCaP cells occurs at the level of BID cleavage. A, LNCaP cells were treated for 6 or 16 h with 1 μg/ml TRAIL, 200 μM wortmannin (WM), or 10 μg/ml cycloheximide (CHX) alone or in combinations. Cell lysates are electrophoresed and consecutively immunoblotted with antibodies specific to caspase 8, XIAP, BID, caspase 9, and caspase 7. The arrow on the left indicates cleavage products. B, LNCaP cells were treated for 6 h with 1 μg/ml TRAIL or 20 μg/ml LY-294002 alone or in combinations. Cell lysates are electrophoresed and consecutively immunoblotted with antibodies specific to BID or the phosphorylated form of Akt (Ser473). C, parental LNCaP cells and LNCaP cells overexpressing Bcl-2 were treated for 6 h with 1 μg/ml TRAIL and 200 μM 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 holocaspase 8 (short exposure) and its 20-kDa proteolytic fragment (long exposure). The arrow indicates caspase 8 cleavage product. D, cell lysates from the experiment described for A were immunoblotted with antibodies that specifically recognize different splice variants of cFLIP protein: FLIPα, FLIPβ, and FLIPδ.
protein kinase is known to block apoptosis (55), we tested whether inhibition of this pathway affects the sensitivity of LNCaP cells to TRAIL. We found that treatment with the PI 3-kinase inhibitors wortmannin and LY-294002 or the protein synthesis inhibitor cycloheximide renders them sensitive to TRAIL-induced apoptosis. 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 (56, 57) 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 proapoptotic factors from mitochondria is essential for TRAIL-induced death of these cells. Scaffidi et al. (30) have proposed that two 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. (58), who argue that the difference between type I and type II cells is an artifact of using agonistic anti-Fas antibodies to trigger Fas signaling instead of Fas ligand. 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 with 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 dysfunction 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 proapoptotic 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 (59). Using immunoblot analysis, we found that cleavage of caspase 8 and one of its substrates, the antiapoptotic protein XIAP (51) 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, and 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 proapoptotic 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 (60). 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 (55). These include direct phosphorylation and modulation of proapoptotic proteins BAD (48) and caspase 9 (50), activation of antiapoptotic NF-κB-mediated transcriptional pathways (61, 62), or phosphorylation of the Forkhead family of transcription factors, preventing them from inducing the transcription of proapoptotic genes (63). 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 significantly reduces the level of cellular cFLIP down-regulating cFLIP. To examine this hypothesis, we tested that protein synthesis inhibitors sensitize cells to TRAIL by exploring 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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