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Tumor Necrosis Factor-related Apoptosis-inducing Ligand-induced Apoptosis of Human Melanoma Is Regulated by Smac/DIABLO Release from Mitochondria[1]

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ABSTRACT

In previous studies we have shown that the sensitivity of melanoma cell lines to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis was determined largely by the level of expression of death receptor TRAIL receptor 2 on the cells. However, approximately one-third of melanoma cell lines were resistant to TRAIL, despite expression of high levels of TRAIL receptor 2. The present studies show that these cell lines had similar levels of TRAIL-induced activated caspase-3 as the TRAIL-sensitive lines, but the activated caspase-3 did not degrade substrates downstream of caspase-3 [inhibitor of caspase-activated DNase and poly(ADP-ribose) polymerase]. This appeared to be due to inhibition of caspase-3 by X-linked inhibitor of apoptosis (XIAP) because XIAP was bound to activated caspase-3, and transfection of XIAP into TRAIL-sensitive cell lines resulted in similar inhibition of TRAIL-induced apoptosis. Conversely, reduction of XIAP levels by overexpression of Smac/DIABLO in the TRAIL-resistant melanoma cells was associated with the appearance of catalytic activity by caspase-3 and increased TRAIL-induced apoptosis. TRAIL was shown to cause release of Smac/DIABLO from mitochondria, but this release was greater in TRAIL-sensitive cell lines than in TRAIL-resistant cell lines and was associated with down-regulation of XIAP levels. Furthermore, inhibition of Smac/DIABLO release by overexpression of Bcl-2 inhibited down-regulation of XIAP levels. These results suggest that Smac/DIABLO release from mitochondria and its binding to XIAP are an alternative pathway by which TRAIL induces apoptosis of melanoma, and this pathway is dependent on the release of activated caspase-3 from inhibition by XIAP and possibly other inhibitor of apoptosis family members.

INTRODUCTION

TRAIL[3] is a member of the TNF family that, like TNF-α and Fas ligand, is a type II membrane protein that can induce apoptotic cell death in a variety of cell types (1–3). TRAIL appears to be particularly important because it can induce apoptosis in a wide range of cultured malignant cells but not in normal tissues (4–9), with the possible exception of human liver cells (10). The potential importance of TRAIL as an anticancer agent has been supported by studies in animal models showing selective toxicity to transplanted human tumors but not to normal tissues (11–13). Induction of apoptosis by TRAIL is believed to be mediated by interaction with two death receptors on cells referred to as TRAIL-R1 and TRAIL-R2 (see review in Ref. 3 for alternate nomenclature). Normal cells were postulated to be protected from TRAIL-induced apoptosis by their expression of TRAIL-R3 and TRAIL-R4, which lack cytoplasmic death domains and act to sequester TRAIL (decoy receptors) or to mediate antiapoptotic signals (6, 14).

We have shown previously that TRAIL, but not other members of the TNF family, was able to induce varying degrees of apoptosis in approximately two-thirds of the melanoma cell lines tested (4, 5). Sensitivity of melanoma cells to TRAIL-induced apoptosis showed an overall correlation with the level of death receptors and TRAIL-R2 expression but did not correlate with the level of expression of the decoy receptors, TRAIL-R3 and TRAIL-R4. Resistance of some cell lines to TRAIL was due to the absence of all receptors for TRAIL, but approximately 30% of cell lines had no or low sensitivity to TRAIL-induced apoptosis despite the presence of death receptors on their surface (5).

These results would be consistent with abnormalities in the caspases or inhibition of their activity by proteins known to act at various levels in the caspase pathway. These include the IAP family, *e.g.*, c-IAP1 and c-IAP2, XIAP, and survivin, which can bind and inhibit caspase-3, caspase-7, and caspase-9 (15, 16). Melanoma cells may also express a protein called cellular FLIP, which prevents interaction of caspase-8 with the adaptor protein Fas-associated death domain and therefore acts at an apical position in membrane-triggered apoptosis (17, 18). The Bcl-2 family is a third group of proteins that act at the level of the mitochondria to prevent release of mitochondrial intermembrane proteins, such as cytochrome *c*, and thereby inhibit apoptosis induced by changes in mitochondria. More recently, a protein released from mitochondria has been described and referred to as Smac/DIABLO (second mitochondrial-derived activator caspase/direct IAP-binding protein with low pI), which promotes caspase activity by binding to IAPs (19, 20).

In the present study, we have examined the extent to which these regulators of apoptosis may determine the sensitivity of human melanoma cells to TRAIL-induced apoptosis. We report that inhibition of TRAIL-induced apoptosis in at least one-third of melanoma cell lines appears to be determined at the level of the effector caspase-3, and this may be regulated by Smac/DIABLO release from mitochondria.

MATERIALS AND METHODS

Cell Lines. Human melanoma cell lines Me4405, Mel-FH, Mel-RM, Mel-CV, MM200, Me1007, Mel-RMu, IgR3, Mel-LT, Mel-AT, Me10538, and SK-Mel-28 have been described previously (4, 5). The cell lines were cultured in DMEM containing 5% FCS (Commonwealth Serum Laboratories, Melbourne, Australia).

Antibodies, Recombinant Proteins, and Other Reagents. Recombinant human TRAIL (lot 6321-19) was supplied by Immunex (Seattle, WA). The preparation was supplied as a leucine zipper fusion protein, which required no further cross-linking for maximal activity. The MAbs against TRAIL-R1 (IgG2a hu TR1-M271; lot 7136-07), TRAIL-R2 (IgG1 hu TRAIL-R2-M413; lot 5274-96), TRAIL-R3 (IgG1 hu TR3-M430; lot 7313-217), and TRAIL-R4 (IgG1 hu TR4-M444; lot 7136-15) were supplied by Immunex and have been described previously (21). The rabbit antiserum against ICAD (Daffne) and the control preimmune serum were a kind gift from Dr. S. L. Sabol (National Cancer Institute, Bethesda, MD) and are described elsewhere (22). The rabbit MAb against activated caspase-3 and rabbit polyclonal Ab anti-caspase-3 were purchased from PharMingen (Bioclone, Marrickville, Australia; catalogue

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[3] The abbreviations used are: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TNF, tumor necrosis factor; TRAIL-R, TRAIL receptor; IAP, inhibitor of apoptosis; c-IAP, cellular inhibitor of apoptosis; XIAP, X-linked IAP; ICAD, inhibitor of caspase-activated DNase; ICAD-L, long form of ICAD; ICAD-S, short form of ICAD; PARP, poly(ADP-ribose) polymerase; FLIP, FLICE/caspase-8-inhibitory protein; MAb, monoclonal antibody; Ab, antibody; PE, phycoerythrin; GFP, green fluorescence protein; CAD, caspase-activated DNase; Act-D, actinomycin D; CHX, cyclohexamide; FLICE, FADD-like interleukin-1 converting enzyme.

numbers 68651G and 65906E, respectively). Rabbit polyclonal Abs against caspase-8 and Bid were also from PharMingen (catalogue numbers 69236E and 68836E, respectively). Rabbit polyclonal Abs against c-IAP1 and c-IAP2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA; catalogue numbers sc-7943 and sc-7944, respectively). Mouse MAb against XIAP was purchased from Transduction Laboratories (Lexington, KY; catalogue number H62120). Anti-PARP p85 fragment was a rabbit polyclonal Ab from Promega (Madison, WI; catalogue number G7341). Rat polyclonal Ab against cellular FLIP was a kind gift from Dr. J. Tschopp (Institute of Biochemistry, University of Lausanne, Epalinges, Switzerland). Rabbit polyclonal Ab against human Smac was a kind gift from Dr. Xiao Dong Wang (Howard Hughes Medical Institute, Dallas, TX), and rat polyclonal Ab against mouse DIABLO was kindly supplied by Dr. David Vaux (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). Annexin V-PE conjugate was purchased from PharMingen (catalogue number 65875X). Isotype control MAbs used were the ID4.5 (mouse IgG2a) MAb against *Salmonella typhi* supplied by Dr. L. Ashman (Institute for Medical and Veterinary Science, Adelaide, Australia), the 107.3 mouse IgG1 MAb purchased from PharMingen (San Diego, CA), and rat IgG and rabbit IgG from Sigma Chemical Co. (Castle Hill, Australia; catalogue numbers 14131 and 15006, respectively).

Plasmid Vectors and Transfection. Expression constructs of pEF Bcl-2, pEF mouse DIABLO, pEF C-FLAG XIAP, and pEGFP were kind gifts from Dr. David Vaux (The Walter and Eliza Hall Institute of Medical Research). These constructs are PEF-puro vectors carrying human Bcl-2, the NH_2-terminal 50 amino acids of mouse DIABLO, C-FLAG human XIAP, and GFP, respectively. Stable transfectants of Bcl-2 were established by electroporation in a gene pulser electroporator (Bio-Rad, Hercules, CA); pEF C-FLAG XIAP was stably transfected into melanoma cells by cationic polymer transfection reagent ExGen 500 (Fermentas, Hanover, MD). Twenty-four h after transfection, cells were selected with puromycin (2 μg/ml) until stable colonies appeared on the plates. Transient transfection was carried out for pEF mouse DIABLO, and the transfection efficiency was visualized by cotransfection with pEGFP at one-twentieth the concentration of DNA. The electroporation was carried out in a gene pulser electroporator (Bio-Rad) with 30 μg of pEF mouse DIABLO and 1.5 μg of pEGFP. Cells were maintained in DMEM containing 10% FCS. Forty-eight h later, cells were subjected to further analysis.

Flow Cytometry. Analysis was carried out using a Becton Dickinson (Mountain View, CA) FACScan flow cytometer, as described previously (5). The percentage of antigen-positive cells was calculated as the difference in positive area between the positive and negative control histograms. The positive area was that to the right of the intersection of the two curves (23).

Apoptosis. Apoptotic cells were determined by the propidium iodide method described elsewhere (4, 5). In some experiments, apoptosis was measured by staining with PE-conjugated annexin V according to the manufacturer's instructions. In brief, cells with or without pretreatment with TRAIL were washed twice with cold PBS and then resuspended in binding buffer at a concentration of 1×10^6 cells/ml. One hundred μl of the resulting solution (1×10^5 cells) were transferred to a 5-ml culture tube, and 5 μl of annexin V-PE was added. After incubation at room temperature for 15 min in the dark, an additional 400 μl of binding buffer were added to each tube, and cells were analyzed by flow cytometry within 1 h. For measurement of apoptosis in DIABLO/Smac transfectants in which GFP was cotransfected, the percentage of annexin V-PE-positive cells was only calculated in the gated GFP-positive population. For measurement of apoptosis in XIAP FLAG transfectant, cells were also stained with the anti-FLAG M2 MAb (Sigma Chemical Co.), which was then labeled with a FITC-conjugated secondary Ab (Silenus; Amrad Biotech, Boronia, Australia). The percentage of annexin V-PE-positive cells was calculated in the gated green fluorescence-positive population.

Immunofluorescence Microscopy. Cells were seeded onto gelatin-coated sterile glass coverslips in 24-well plates (Falcon 3047; Becton Dickinson, Lane Cove, Australia) 16–24 h before fixation and treated with or without TRAIL (100 ng/ml) for the indicated time periods. The cells were washed in PBS, fixed in 2% paraformaldehyde for 5 min, and permeabilized with 0.1% saponin in PBS containing 10% human AB serum for 10 min. Cells were then incubated with primary Abs diluted in PBS containing 1% human AB serum at 4°C for 45 min. After washing with PBS, cells were incubated with Oregon Green 488 goat antimouse IgG conjugate (Molecular Probes, Eugene, OR; catalogue number O-11000) or FITC-conjugated sheep antimouse (Silenus; catalogue number 985051020) secondary Abs at 4°C for 45 min. For double labeling, cells were washed and incubated with cellular organelle-specific fluorescent reagents at 4°C for 30 min. Coverslips were mounted in Gel-Mount (Biomeda, Foster, CA) and examined using a Zeiss Axiophot microscope (Oberkochem, Germany).

Western Blot Analysis. Methods used were as described previously, with minor modification (5). Briefly, the protein content of cell extracts was determined by the Bradford assay (Bio-Rad). A total of 20–30 μg of protein were electrophoresed on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked, incubated with primary Abs at the appropriate concentration, and subsequently incubated with horseradish peroxidase-conjugated goat antirabbit IgG (1:3000 dilution; Bio-Rad; catalogue number 170-6518). Labeled bands were detected by Renaissance Western Blot Chemiluminescence Regent (New England Nuclear Life Science Products, Boston, MA) and exposed on Hyper MP autoradiography film (Amersham). Whole cell extracts were obtained by lysing cells in a Triton X-100-based lysis buffer [10% Triton X-100, 10% glycerol, 150 mM NaCl, 20 mM Tris (pH 7.5), 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin].

Preparation of Mitochondrial and Cytosolic Fractions. Methods used for subcellular fraction were similar to the methods described by others (24). Adherent cells were removed by trypsinization in 0.25% trypsin at 37°C for 5 min. When cells were pretreated with TRAIL, floating cells were also collected in the same tubes, after being washed once with ice-cold PBS, the cell pellet was suspended in 5 volumes of buffer A [20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM Na-EDTA, 1 mM Na-EGTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride containing 250 mM sucrose] supplemented with protease inhibitors (5 μg/ml pepstatin A, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 25 μg/ml *N*-acetyl-leu-leu-norleucine (ALLN)). After incubation on ice for 15 min, the cells were disrupted by passing them 15 times through a 22-gauge needle. After centrifugation twice at 750 $\times$ *g* for 10 min at 4°C, the supernatant was collected and centrifuged at 10,000 $\times$ *g* for 15 min at 4°C, and the resulting mitochondrial pellets were resuspended in buffer A. The supernatants of the 10,000 $\times$ *g* spin were further centrifuged at 100,000 $\times$ *g* for 1 h at 4°C, and the resulting supernatants were designated as the S-100 cytosolic fraction.

Immunoprecipitation. After cells were lysed for whole cell extracts, the nuclear debris and cellular debris were cleared by centrifugation. The resulting lysates (100 μl) were precleared by incubation with 50 μl of protein G-Sepharose-packed beads (Amersham) in a rotator at 4°C for 2 h, followed by incubation with 50 μl of freshly packed beads in a rotator at 4°C overnight. Anti-XIAP MAb (20 μg) was then added to the lysate, which was rotated at 4°C for 2 h. The beads were then pelleted by centrifugation and washed four times with ice-cold lysate buffer before elution of the proteins from the beads in lysate buffer at room temperature for 1 h. The resulting immunoprecipitates were then subjected to SDS-PAGE and Western blot analysis.

Immunodepletion of XIAP. An aliquot of 100 μl (250 μg/ml) of anti-XIAP MAb was incubated with 50 μl of protein G-agarose beads resuspended in 100 μl of PBS in a rotator at 4°C for 3 h. The beads were collected by centrifugation for 15 min in a microcentrifuge at 4°C. After removal of the supernatant, the beads were washed once with 1 ml of cell lysis buffer and incubated with 1 ml of whole cell lysates for 6 h in a rotator at 4°C. The beads were subsequently pelleted by centrifugation for 15 min in a microcentrifuge at 4°C. The supernatant was used as whole cell extracts immunodepleted of XIAP.

RESULTS

TRAIL Induces Caspase-3 Activation in Death Receptor-expressing Melanoma Cells That Are Resistant to TRAIL-induced Apoptosis. To study the role of caspase-3 activation in TRAIL-induced apoptosis of melanoma cells, we measured activated caspase-3 in cells with or without exposure to TRAIL using a MAb that specifically recognizes proteolytically cleaved caspase-3. Representative flow cytometric histograms for Me4405 and Mel-RMu cells are shown in Fig. 1*A*. A summary of representative studies on at least two occasions on a panel of melanoma cell lines is shown in Table 1. Caspase-3 activation induced by TRAIL is detected to a varying degree in all melanoma cell lines expressing the death receptors

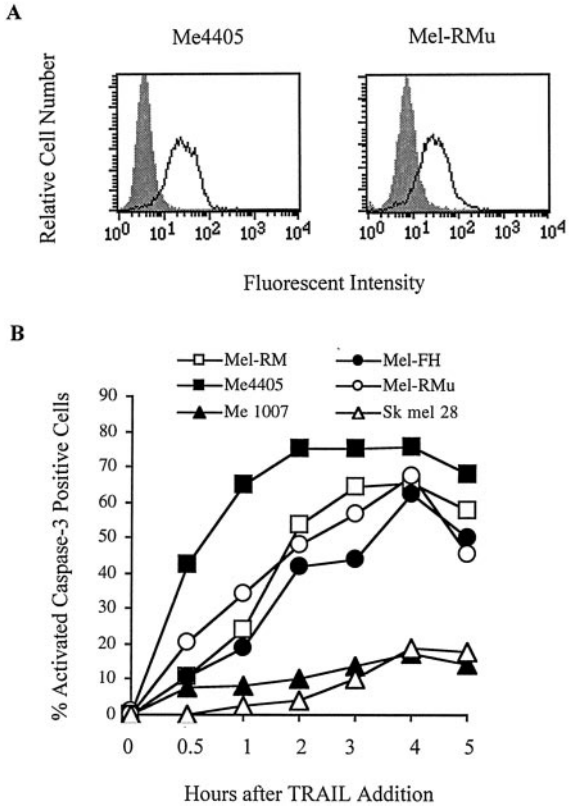

Fig. 1. *A*, expression of activated caspase-3 in melanoma cells after treatment with TRAIL. Cells were treated with 100 ng/ml TRAIL at 37°C for 3 h before fixation. *Filled histograms* are isotype controls. *B*, kinetics of caspase-3 activation induced by TRAIL. Cells were treated with 100 ng/ml TRAIL for the indicated time periods. Caspase-3 activation was measured by flow cytometry. Data indicate the percentage of positive cells.

Table 1 *Summary of surface TRAIL-R2 expression, caspase-3 activation, and apoptosis induced by TRAIL in melanoma cell lines*[a]

Cell lines	% TRAIL-R2	% Activated caspase-3	% Apoptosis
Mel-CV	72	75.6 (12.4)	54
Mel-RM	59.6	65.37 (6.6)	76
Mel-FH	57.2	62.3 (2.9)	24
Me4405	88.4	86.5 (8.4)	61
MM200	76.1	74.2 (7.2)	83
Me1007	10.4	17.39 (1.8)	0
SK-Mel-28	18.6	18.8 (2.2)	20
Mel-RMu	50.6	67.7 (4.9)	15
IgR3	79.9	71.2 (4.1)	21
Mel-LT	63.4	50.1 (2.01)	15
Mel-AT	40.2	8.4 (1.6)	0
Me10538	0	9.2 (1.1)	0

[a] Values indicate the percentage of positive cells. Values in parentheses indicate the mean fluorescence intensity.

TRAIL-R1 and/or TRAIL-R2 except Mel-AT. In contrast, Me10538, which lacks death receptors for TRAIL, showed only the basal level of activated caspase-3 after treatment with TRAIL. The kinetics of caspase-3 activation in representative melanoma cell lines is shown in Fig. 1*B* and indicates that activated caspase-3 was detectable as early as 30 min after TRAIL treatment, with peak activation ranging from 2–4 h between different cell lines.

Our previous studies have shown that the level of expression of TRAIL death receptors, in particular, TRAIL-R2, appeared to be an important determinant of the level of TRAIL-induced apoptosis in melanoma cell lines (5). Fig. 2, *A* and *B*, shows that cell surface TRAIL-R2 expression was correlated even more closely with the proportion of cells expressing TRAIL-induced activated caspase-3. Fig. 2*C* illustrates the correlation of the latter with apoptosis induced by TRAIL. For most melanoma cell lines, there was a good correlation between the two, but in several cell lines, TRAIL induced low levels of apoptosis despite inducing caspase-3 activation in a high proportion of cells (Mel-FH, Mel-RMu, IgR3, and Mel-LT; see Table 1). Thus, TRAIL induces caspase-3 activation but not apoptosis in a subpopulation of melanoma cells that express TRAIL-R2 death receptors on their surface.

These results did not appear to be due to a requirement for higher concentrations of TRAIL to induce apoptosis in the resistant cells because resistance to TRAIL was seen over a wide range of TRAIL concentrations, even though activation of caspase-3 was increased at the higher concentrations of TRAIL (Fig. 3).

TRAIL-induced Apoptosis Is Correlated with TRAIL-induced Changes in the ICAD and PARP. We examined whether the activated caspase-3 was proteolytically active by evaluating whether some of its known substrates were degraded. One of the substrates is a DNase referred to as CAD (DFF40/CPAN), which is directly responsible for the characteristic fragmentation of DNA (25, 26). CAD is complexed with its inhibitor, which is referred to as ICAD (DFF45), but during apoptosis, ICAD is proteolytically degraded by activated caspase-3, releasing active CAD (25, 26). We examined ICAD expression in melanoma cells in relation to TRAIL treatment. As shown in Fig. 4*A*, both ICAD-L and ICAD-S were detected in extracts of melanoma cells, with ICAD-L being more abundantly expressed than

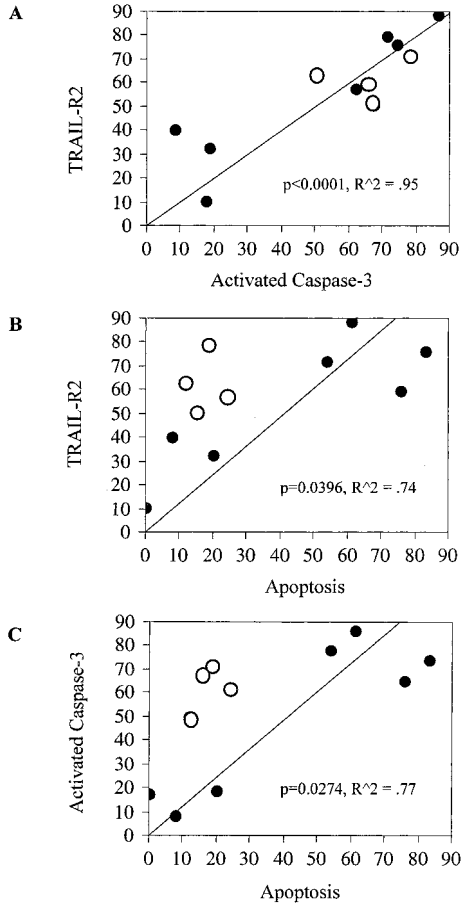

Fig. 2. Regression analysis of the relation between surface TRAIL-R2 expression and TRAIL-induced caspase-3 activation (*A*), TRAIL-R2 expression and TRAIL-induced apoptosis (*B*), and TRAIL-induced caspase-3 activation and apoptosis (*C*). Data are the percentage of positive cells measured by flow cytometry. *Open circles* are cells with high levels of surface TRAIL-R2 expression and TRAIL-induced caspase-3 activation but low levels of TRAIL-induced apoptosis (Mel-RMu, Mel-FH, Mel-LT, and IgR3; see Table 1).

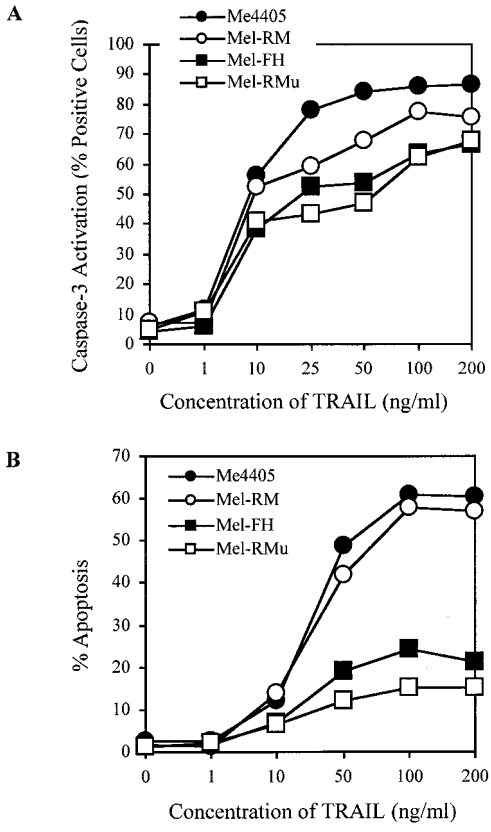

Fig. 3. Caspase-3 activation and apoptosis induced by different doses of TRAIL. *A*, melanoma cells were treated with the indicated doses of TRAIL at 37°C for 3 h before fixation. Data indicate the percentage of activated caspase-3-positive cells. *B*, melanoma cells were treated with the indicated doses of TRAIL at 37°C for 20 h before DNA fragmentation was measured using the propidium iodide method. Data are the percentage of apoptotic cells.

ICAD-S. After exposure to 100 ng/ml TRAIL for 3 h, both ICAD-L and ICAD-S fractions were barely detected in extracts from the TRAIL-sensitive melanoma cell lines (Me4405 and Mel-RM; data not shown), whereas there was only a minor reduction in expression in the TRAIL-insensitive cell lines Mel-FH and Mel-RMu. The level of ICAD in the TRAIL-resistant cell line Mel-AT appeared unaltered after treatment with TRAIL (Fig. 4*B*). Fig. 4*C* shows that cleavage of ICAD was detectable at 30 min after exposure to TRAIL, similar to the kinetics of activation of caspase-3.

Another substrate for activated caspase-3 is PARP. We measured the cleavage of PARP in whole cell extracts from melanoma cells before and after treatment with TRAIL, using a MAb that recognizes only the p85 fragment of cleaved PARP. As expected, the p85 fragment was readily detected in the TRAIL-sensitive Me4405 cells (and in Mel-RM cells; data not shown) but was barely detectable in extracts from the TRAIL-insensitive Mel-FH and Mel-RMu cells. Cleaved PARP was not detected in Mel-AT cells after exposure to TRAIL (Fig. 4*D*). The results for studies on Mel-RM were similar to those for Me4405 and were omitted to assist clarity of the results in the figure.

Taken together, these results suggest that inhibition of TRAIL-induced apoptosis in the TRAIL-resistant melanoma cell lines occurred upstream of processing of ICAD/CAD and PARP, most probably at the level of activated caspase-3.

TRAIL Induces Equivalent Cleavage of Caspase-8 and Bid in TRAIL-sensitive and -resistant Melanoma Cell Lines That Express TRAIL Death Receptors. Although these results suggested that the regulation of TRAIL-induced apoptosis was occurring at the level of activated caspase-3, we examined whether events upstream of caspase-3 may be involved. Caspase-8 is thought to be the primary apical caspase involved in the induction of apoptosis by TRAIL (27). Fig. 5*A* shows that caspase-8 was expressed in all melanoma cell lines tested except for Me1007. Me1007 was subsequently shown by PCR to lack mRNA for caspase-8 (data not shown). The levels of expression in TRAIL-sensitive melanoma cell lines appeared to be slightly higher than those seen in insensitive melanoma cell lines, but after exposure of cells to 100 ng/ml TRAIL for 3 h, equivalent cleavage of caspase-8 into smaller forms was seen in all of the cell lines expressing TRAIL death receptors except Mel-AT (Fig. 5*B*). The latter cell line showed no change in caspase-8 expression before and after exposure to TRAIL. As shown in Fig. 5*C*, cleavage of caspase-8 was detected as early as 30 min after TRAIL treatment and reached a peak at 3 h after TRAIL treatment.

One of the substrates for activated caspase-8 is Bid, which was shown by others (28) to induce proapoptotic changes in mitochondria and shown by us to be involved in TRAIL-induced apoptosis of melanoma (29). Fig. 5*D* shows that TRAIL induced similar processing of Bid in melanoma cells irrespective of whether they were sensitive or insensitive to TRAIL-induced apoptosis. Me1007, which lacks caspase-8, showed no Bid expression. Taken together, these

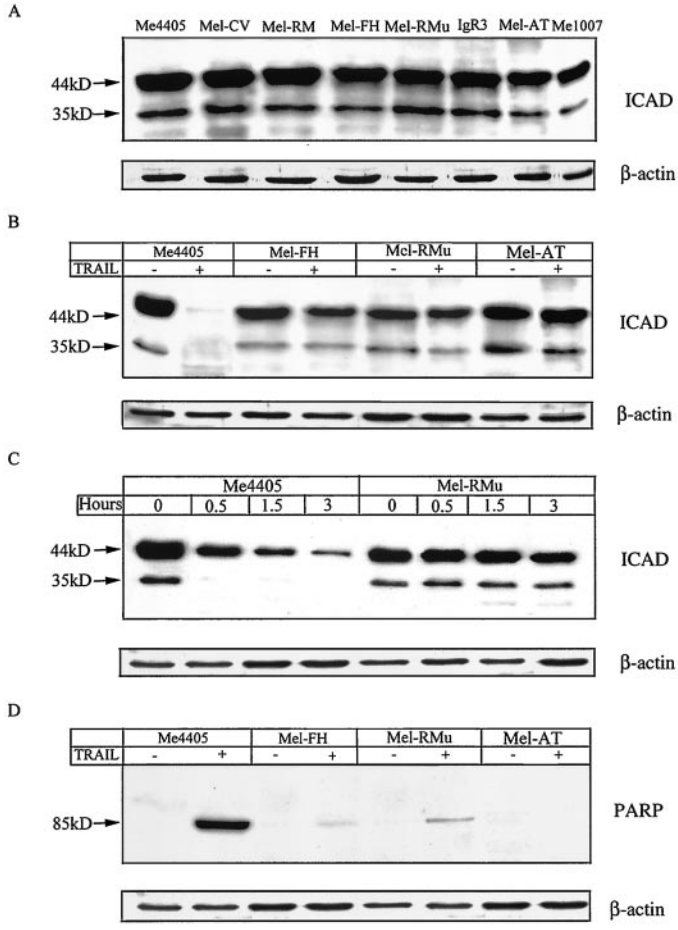

Fig. 4. *A*, expression of ICAD in melanoma cells. Whole cell lysates were subjected to Western blot analysis. *B*, TRAIL induced ICAD cleavage in melanoma cells. Whole cell extracts from cells with or without TRAIL pretreatment at 37°C for 3 h were subjected to Western blot analysis. *C*, time course of cleavage of ICAD induced by TRAIL. Cells were treated for the indicated time periods with 100 ng/ml TRAIL at 37°C before whole cell lysates were extracted. *D*, cleavage of PARP by TRAIL. Whole cell lysates from melanoma cells with or without pretreatment with 100 ng/ml TRAIL at 37°C for 3 h were subjected to Western blot analysis. Western blot analysis of β-actin levels was included to show that equivalent amounts of protein were loaded in each lane.

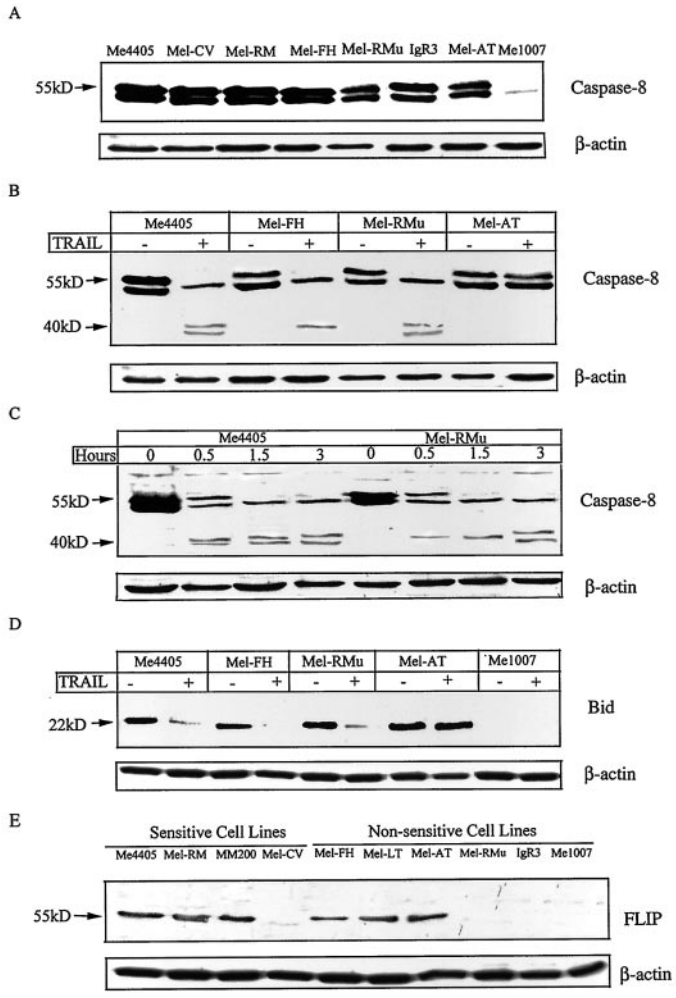

Fig. 5. *A*, caspase-8 expression in whole cell extracts of melanoma cells. Note that there is no caspase-8 expression in cell lysates from Me1007. *B*, cleavage of caspase-8 by TRAIL in whole cell lysates of melanoma cells before and 3 h after treatment with 100 ng/ml TRAIL at 37°C. *C*, kinetics of TRAIL-induced caspase-8 cleavage. Whole cell lysates from melanoma cells were treated with TRAIL (100 ng/ml) for the time periods shown at 37°C. *D*, cleavage of Bid by TRAIL in whole cell lysates of melanoma cells with or without treatment with 100 ng/ml TRAIL at 37°C for 3 h. Note that there is no Bid expression in cell lysates from Me1007. *E*, FLIP expression in melanoma cells. Whole cell extracts of melanoma cells were subjected to Western blot analysis.

results suggest that regulation of TRAIL-induced apoptosis in these melanoma cell lines occurred downstream of caspase-8 and Bid.

FLIP levels were also assessed in Western blots, but, as reported previously (5), there was no correlation with sensitivity to TRAIL. Three TRAIL-sensitive cell lines had strong FLIP expression, and three of six TRAIL-resistant cell lines had levels of expression similar to those in the TRAIL-sensitive lines (Fig. 5*E*).

XIAP Binds Cleaved Forms of Caspase-3. One of the IAP family members, XIAP, is known to bind to and inactivate activated caspase-3 (15, 16). We examined whether this may occur in melanoma cells by immunoprecipitation of XIAP from whole cell lysates prepared from the TRAIL-sensitive Me4405 cells and the TRAIL-resistant Mel-RMu cells 3 h after exposure to TRAIL. Analysis of the precipitates for caspase-3 on immunoblots with a MAb against caspase-3 revealed that the p20 fragment of cleaved caspase-3 (30) was readily detected in extracts of both cell lines (Fig. 6*A*). In contrast, the p17 subunit, which is cleaved from the p20 subunit by caspase-3 (30, 31), was detected mainly in precipitates from Me4405, and little, if any, of this subunit was observed in precipitates from Mel-RMu (Fig. 6*A*).

To confirm the association between XIAP and activated caspase-3 in melanoma cells exposed to TRAIL, cell lysates from TRAIL-treated Me4405 and Mel-RMu cells were immunodepleted of XIAP and analyzed for activated caspase-3 subunits. As shown in Fig. 6*B*, immunodepletion of XIAP removed most of the p20 fragment of cleaved caspase-3 but had little effect on the p17 subunits. p17 was again not detected in lysates from Mel-Rmu cells.

Overexpression of XIAP Protects Melanoma Cells from TRAIL-induced Apoptosis. To confirm that XIAP could inhibit TRAIL-induced apoptosis of melanoma cells, we transfected Me4405 cells with cDNA encoding FLAG-tagged XIAP. TRAIL-induced apoptosis of XIAP transfectants was measured by flow cytometry using

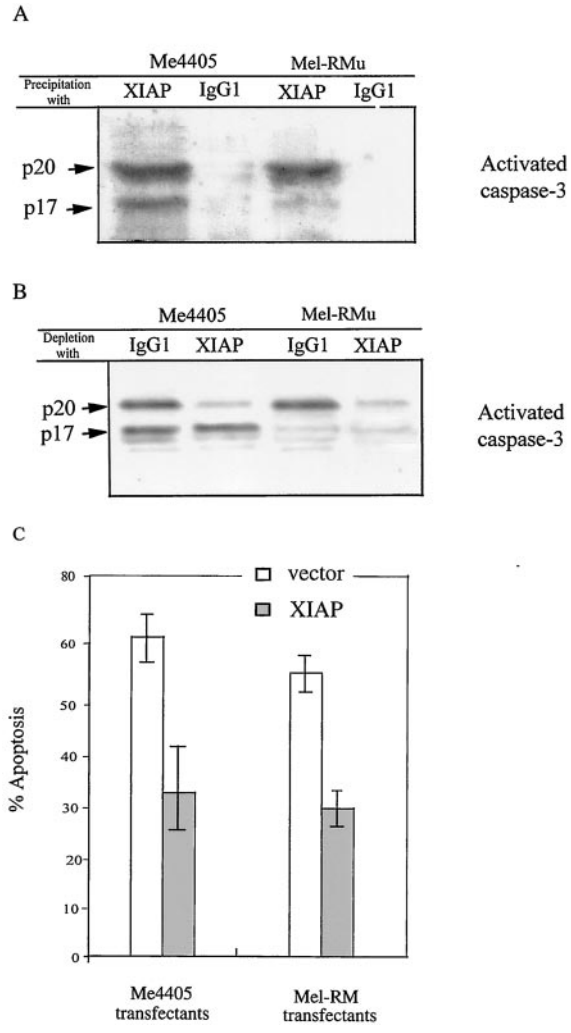

Fig. 6. *A* and *B*, association between XIAP and TRAIL-induced activated caspase-3. *A*, whole cell lysates from melanoma cells treated with 100 ng/ml TRAIL at 37°C for 3 h were subjected to immunoprecipitation with a mouse MAb against XIAP. Purified mouse IgG was used as a control. The precipitates were subjected to SDS-PAGE and probed with MAb against caspase-3. *B*, whole cell lysates from melanoma cells treated with 100 ng/ml TRAIL at 37°C for 3 h were subjected to immunodepletion with a MAb against XIAP or the control purified normal mouse IgG. The depleted extracts were subjected to SDS-PAGE and probed with MAb against caspase-3. *C*, overexpression of XIAP protects melanoma cells from TRAIL-induced apoptosis. Stable transfectants of Me4405 cells were obtained by transfection with pEF C-FLAG XIAP. Forty-eight h later, cells were treated with 100 ng/ml TRAIL at 37°C for 3 h. Melanoma cells were double-labeled with PE-conjugated annexin V and FITC-conjugated anti-FLAG MAb and then subjected to analysis by flow cytometry. Annexin V-PE expression was measured on the cells identified as FITC positive. The data shown are the mean ± SE of three individual experiments. Apoptosis is shown as a percentage of annexin V-positive cells. Control values in the absence of TRAIL in an individual experiment were subtracted from the results (5.8 ± 1.5% and 8.0 ± 1.6% for Me4405 transfected with vector alone and XIAP, respectively; 11.5 ± 2.5% and 7.6 ± 1.8% for Mel-RM transfected with vector alone and XIAP, respectively).

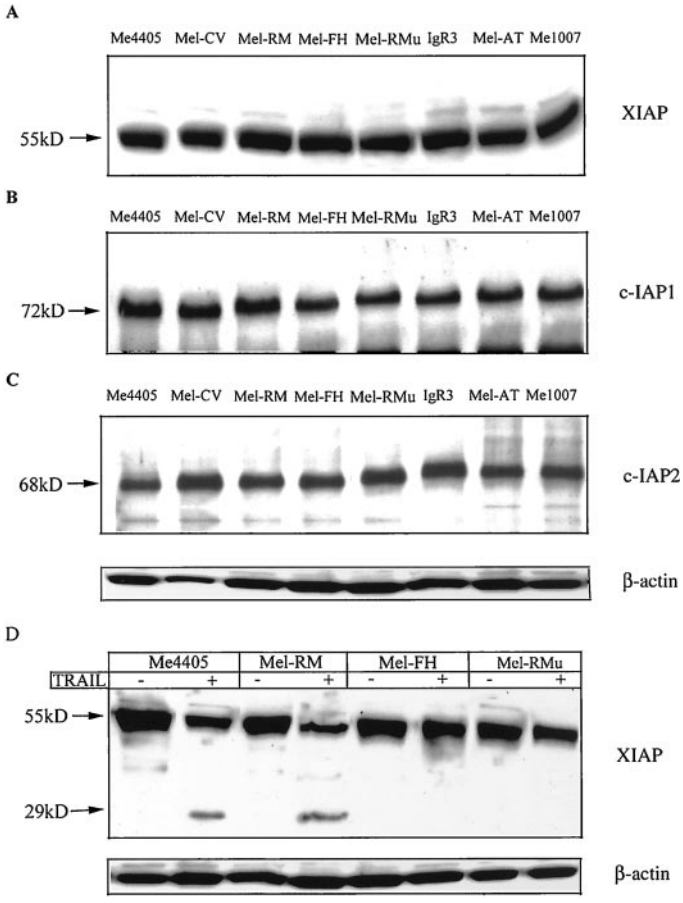

Fig. 7. *A–C*, expression of XIAP (*A*), c-IAP1 (*B*), and c-IAP2 (*C*) in melanoma cells. Whole cell extracts of melanoma cells were subjected to Western blot analysis. *D*, down-regulation of XIAP protein level after treatment of melanoma cells with 100 ng/ml TRAIL at 37° for 3 h. Whole cell lysates from melanoma cells were subjected to Western blot analysis for XIAP expression.

dual staining with PE-conjugated annexin V and FITC-conjugated anti-FLAG MAb. The levels of TRAIL-induced apoptosis in XIAP transfectants were markedly decreased in comparison with those in cells transfected with vector alone (Fig. 6*C*). To confirm that inhibition of apoptosis in XIAP transfectants took place at the level of caspase-3 activation, XIAP was immunoprecipitated with anti-FLAG MAb coupled with agarose beads, and the immunoprecipitates were examined for the presence of activated caspase-3 by Western blotting analysis. As before, activated caspase-3 was detected in the whole cell lysate in two forms, the p20 and p17 fragments (data not shown).

XIAP Is Down-Regulated during TRAIL-induced Apoptosis. Fig. 7, *A–C*, shows that the protein levels of XIAP, IAP1, and IAP2 were in a similar range in melanoma cells, irrespective of their sensitivity to TRAIL, *i.e.*, the constitutive expression of these proteins could not explain differences in sensitivity to TRAIL. Previous studies have shown that XIAP may be down-regulated during apoptosis induced by Fas ligand (32, 33). We examined whether TRAIL may induce similar changes by measuring XIAP expression in permeabilized melanoma cells before and after exposure to TRAIL. Representative flow cytometric histograms for Me4405 and Mel-RMu are shown in Fig. 8*A*, and a summary of studies in a panel of melanoma cell lines is shown in Fig. 8*B*. After treatment with TRAIL, the number of XIAP-positive cells was markedly decreased in TRAIL-sensitive melanoma cell lines (Me4405 and Mel-RM). The reduction was detected as early as 30 min after the addition of TRAIL (Fig. 8*C*). In contrast, the levels of XIAP expression in nonsensitive melanoma cell lines (Mel-FH, Mel-RMu, Mel-LT, and IgR3) were only slightly reduced, and there was no change in XIAP expression in the Mel-AT cell line, which is completely resistant to TRAIL. Cleavage of XIAP during TRAIL-induced apoptosis of melanoma cell lines was confirmed by Western blot analysis as shown in Fig. 7*D*. As reported by others, a p29 band of cleaved XIAP was detected in lysates from TRAIL-sensitive cell lines pretreated with TRAIL (32).

Depletion of XIAP Activates Caspase-3. To obtain further evidence that XIAP inhibited caspase-3, we depleted XIAP levels by treating the melanoma cells with Act-D. As shown in Fig. 9*A*, XIAP was depleted by pretreatment of the melanoma cells with Act-D or CHX for 20 h (IAP1 and IAP2 levels were unchanged). This was associated with the appearance of the p17 form of caspase-3 in the lysates of Mel-RMu and the loss of the p20 form (Fig. 9*B*), indicating that caspase-3 was now catalytically active in the Mel-RMu cells (Fig. 10*B*). These results are consistent with the view that conversion of the p20 fragment of cleaved caspase-3 by autocatalysis to the smaller p17

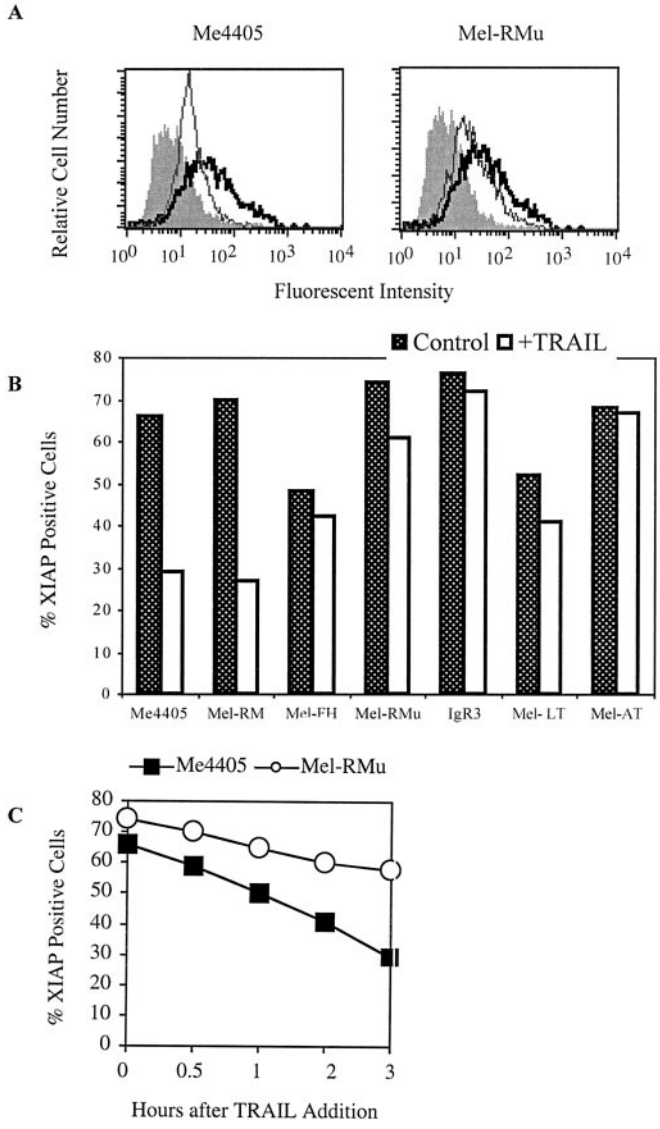

Fig. 8. Down-regulation of XIAP expression levels by TRAIL. *A*, representative flow cytometric histograms of melanoma cells with (*thin lines*) or without (*thick lines*) treatment with 100 ng/ml TRAIL for 3 h. The *filled histograms* are isotype controls. *B*, summary of XIAP expression in a panel of melanoma cell lines with or without pretreatment with 100 ng/ml TRAIL at 37°C for 3 h. Data indicate the percentage of XIAP-positive cells. *C*, kinetics of down-regulation of XIAP by TRAIL. Me4405 and Mel-RMu cells were treated with 100 ng/ml TRAIL at 37°C for 3 h before analysis by flow cytometry. Data indicate the percentage of XIAP-positive cells.

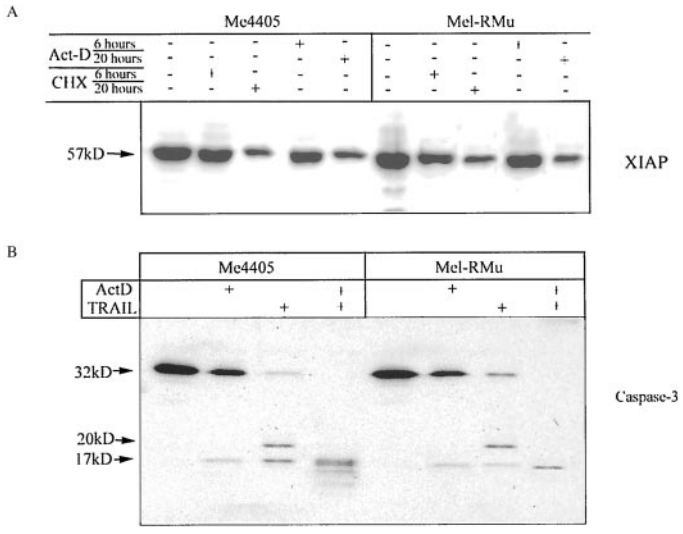

Fig. 9. *A*, down-regulation of XIAP expression by metabolic inhibitors. Whole cell lysates from Me4405 and Mel-RMu cells treated with Act-D or CHX for the indicated times were subjected to Western blot analysis for XIAP expression. *B*, TRAIL-induced caspase-3 activation is enhanced by pretreatment with Act-D. Me4405 and Mel-RMu cells were treated with or without Act-D (0.1 μg/ml) at 37°C for 20 h before adding TRAIL (100 ng/ml) for another 3 h. Whole cell lysates were then analyzed for activated caspase-3 by Western blot analysis.

subunit was accelerated by down-regulation of XIAP levels by pretreatment with Act-D. Pretreatment with Act-D or CHX for 20 h did not reduce IAP1 and IAP2 levels or Bcl-2 and Bcl-X_L levels (data not shown).

TRAIL-induced Relocation of Smac/DIABLO from Mitochondria to Cytosol Is Greater in TRAIL-sensitive than in TRAIL-resistant Melanoma Cell Lines. A possible explanation for the results above showing comparable levels of XIAP before TRAIL treatment but markedly reduced levels after exposure to TRAIL (in TRAIL sensitive lines) is that XIAP levels were regulated by a third factor induced by TRAIL. Smac/DIABLO is a mitochondrial protein that is released into the cytosol during UV-induced apoptosis, where it binds to and inhibits the activity of the IAP family members (19, 20). We therefore examined the expression of Smac/DIABLO in the cell lines before and after exposure to TRAIL. Fig. 10*A* shows that Smac/DIABLO was present at similar levels in extracts of all of the melanoma cell lines in the study before the addition of TRAIL. Mitochondrial localization of Smac/DIABLO in melanoma cells was confirmed by immunostaining of living cells, which showed the typical punctate staining pattern of location within mitochondria (Fig. 10*B*). A similar pattern was found in cells stained with anti-cytochrome *c* MAbs (data not shown).

After exposure of Me4405 and Mel-RMu cells to TRAIL for various time periods, the distribution of Smac/DIABLO changed from the punctate mitochondrial pattern to a more diffuse cytosolic pattern.

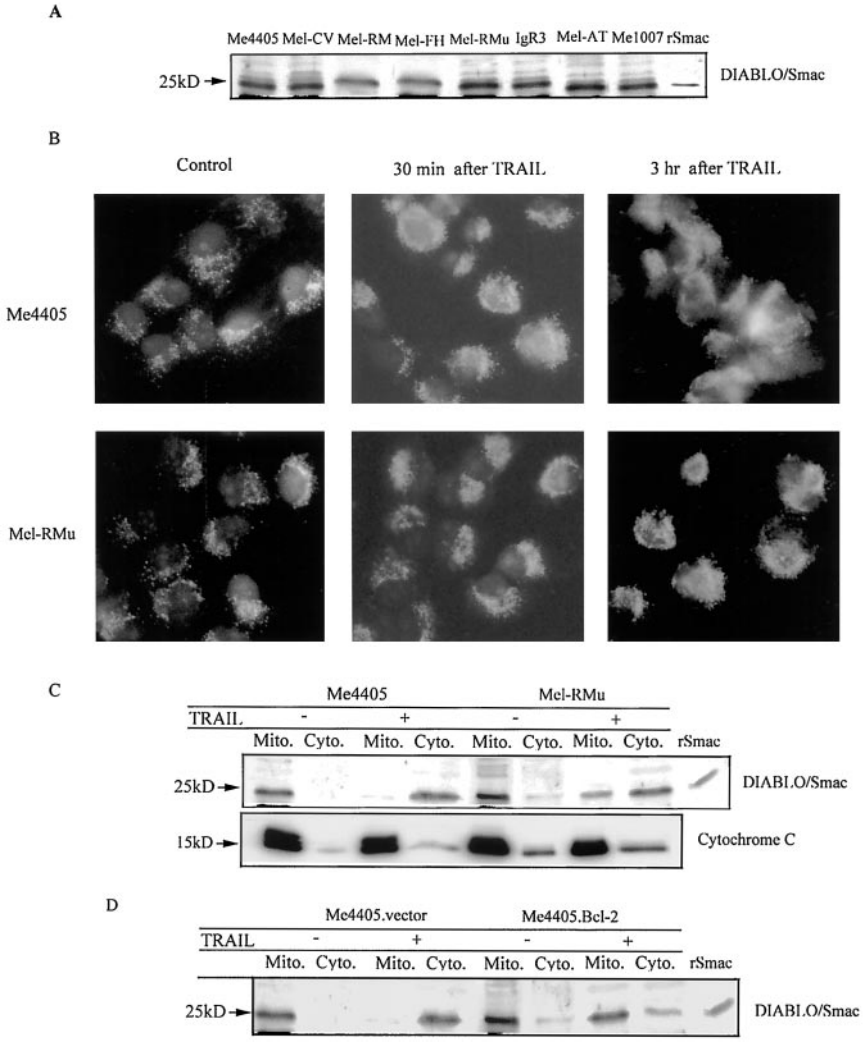

Fig. 10. *A*, expression of Smac/DIABLO in whole cell lysates of melanoma cells. *B*, mitochondrial localization or Smac/DIABLO and release of Smac/DIABLO from mitochondria to cytosol induced by TRAIL. Me4405 and Mel-RMu cells were grown on coverslips and treated with or without 100 ng/ml TRAIL at 37°C for the time periods shown. The cells were fixed, and localization of Smac/DIABLO was examined by fluorescence microscopy. *C*, mitochondrial and cytosolic localization of Smac/DIABLO and cytochrome *c* before and after exposure to TRAIL. Mitochondrial and cytosolic fractions were prepared from Me4405 and Mel-RMu cells that had been treated or not treated with 100 ng/ml TRAIL at 37°C for 3 h and subjected to Western blot analysis. *D*, overexpression of Bcl-2 inhibits TRAIL-induced release of Smac/DIABLO from mitochondria. Me4405 melanoma cells were stably transfected with vector alone or with Bcl-2, and the cells were treated with 100 ng/ml TRAIL at 37°C for 3 h. Mitochondrial and cytosolic fractions were prepared and subjected to Western blot analysis for Smac/DIABLO.

Redistribution of Smac/DIABLO from the mitochondria to the cytosol was more prominent in the TRAIL-sensitive Me4405 cells than in the TRAIL-resistant Mel-RMu cells (Fig. 10*B*). This was observed 30 min after the addition of TRAIL and became more pronounced by 2–3 h (Fig. 10*B*). Cytochrome *c* release was not detectable earlier than 2 h after exposure to TRAIL (data not shown).

To further confirm the immunostaining results, we isolated mitochondrial and cytosolic fractions from two TRAIL-sensitive cell lines, Me4405 and Mel-RM, and two TRAIL-resistant cell lines, Mel-RMu and Mel-FH. As shown in Fig. 10*C*, before exposure to TRAIL, Smac/DIABLO was localized exclusively in mitochondrial fractions. Three h after treatment with TRAIL, Smac/DIABLO was observed in the cytosolic fraction, and there was a corresponding decrease in the mitochondrial fraction. However, Smac/DIABLO was still retained in mitochondrial fractions from Mel-RMu and Mel-FH but not in fractions from Me4405 and Mel-RM, suggesting that release of Smac/DIABLO was incomplete from the mitochondria of Mel-RMu cells. The density of the bands was quantitated on a Macintosh computer using the public domain NIH Image program, which is available on the internet.[4] When the fraction of Smac/DIABLO in the cytosol was expressed as a percentage of mitochondrial plus cytosol fractions, the percentage of Smac/DIABLO released by TRAIL in Me4405 and Mel-RM cells was 97% and 94%, respectively, as compared with 62% and 63%, respectively, in the TRAIL-resistant Mel-RMu and Mel-FH cells. Over the same time period, there was negligible TRAIL-mediated release of cytochrome *c* from the mitochondria of the two melanoma cell lines (Fig. 10*C*). There was also no evidence for activation of caspase-9 at this time, as assessed by MAbs against the activated form of caspase-9 (data not shown).

Smac/DIABLO release was dependent on mitochondrial membrane permeability induced by TRAIL and was markedly reduced in melanoma cells in which Bcl-2 was overexpressed. This is shown in Fig. 10*D*, where the majority of the Smac/DIABLO was retained in the mitochondrial fraction in cells transfected with Bcl-2, but TRAIL induced almost complete release of Smac/DIABLO in the nontransfected cells.

Overexpression of Smac/DIABLO in TRAIL-resistant Melanoma Enhances Caspase-3 Activity and Increases TRAIL-induced Apoptosis. To further investigate the role of Smac/DIABLO in melanoma, we overexpressed Smac/DIABLO by transfection into the TRAIL-resistant cell lines Mel-RMu and Mel-FH. The transfected cells were cultured for 48 h and exposed to 100 ng/ml TRAIL for 3 h. As indicated in Fig. 11, overexpression of Smac/DIABLO was associated with TRAIL-mediated disappearance of the p20 form of caspase-3 and degradation of one of its substrates, ICAD, indicating that the activated form of caspase-3 was now catalytically active. This was associated with an increase in TRAIL-mediated apoptosis, as measured in the annexin V assay. Similar results were obtained in a repeat of the assay on Mel-RMu cells and in studies on Smac/DIABLO-transfected Mel-FH cells.

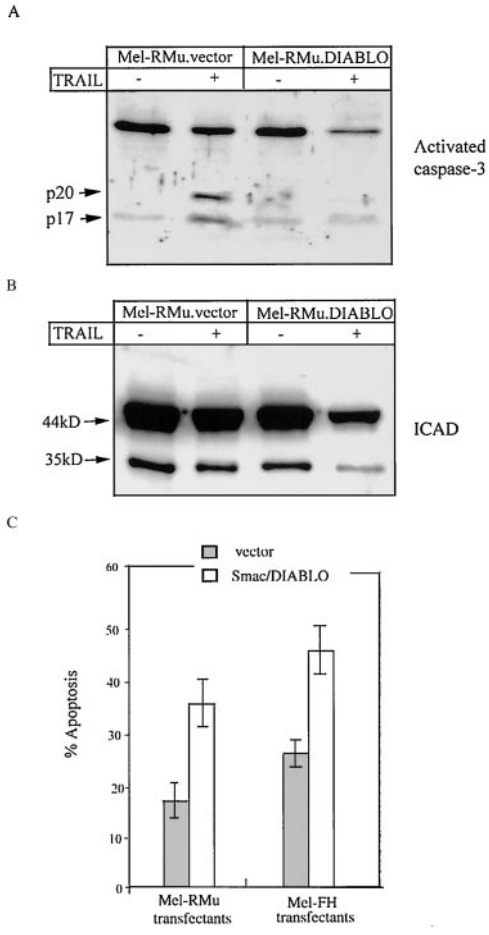

Fig. 11. Overexpression of Smac/DIABLO enhances caspase-3 activity and increases TRAIL-induced apoptosis. Mel-RMu cells were transiently cotransfected with pEF mouse DIABLO and pEGFP or with the vector alone. Cells were grown for 48 h after transfection and exposed to 100 ng/ml TRAIL for 3 h. *A*, activated caspase-3 levels in whole cell lysates of the transfected cells. *B*, ICAD levels in whole cell lysates from the transfectants. *C*, apoptosis measured by annexin V-PE expression on cells gated for green fluorescence emission. The data shown are the mean ± SE of results from three individual experiments. Control values in the absence of TRAIL in an individual experiment were subtracted from the results (9.8 ± 2.5% and 12.4 ± 2.6% for Mel-RMu transfected with vector alone and Smac/DIABLO, respectively; 10.6 ± 3.0% and 12.4 ± 2.9% for Mel-FH transfected with vector alone and Smac/DIABLO, respectively).

DISCUSSION

We have shown in previous studies that the sensitivity of melanoma cells to TRAIL-induced apoptosis was determined largely by the level of expression of the death receptor TRAIL-R2 (5) and that apoptosis was dependent on recruitment of the mitochondrial pathway to apoptosis (29). In the present study, we show that a much closer correlation exists between the expression of TRAIL-R2 and the levels of activated caspase-3 in the melanoma cells. This is because some melanoma cells with high TRAIL-R2 and high TRAIL-activated caspase-3 levels were resistant to TRAIL-induced apoptosis. Four of 11 melanoma cell lines were identified that fitted into this category. The presence of high levels of activated caspase-3 implied that the apoptotic pathway was inhibited at the level of caspase-3 itself or downstream of caspase-3. Moreover, it seemed unlikely that inhibitors upstream of caspase-3, such as FLIP, were playing a significant role because they would prevent activation of caspase-3. These conclusions were supported by studies showing comparable levels of TRAIL-induced degradation of caspase-8 and Bid in melanoma lines that were sensitive or resistant to TRAIL-induced apoptosis and were consistent with our previous finding that FLIP expression did not appear to be related to the resistance of melanoma cells to TRAIL (5). These results were confirmed in the present study.

Despite similar levels of TRAIL-activated caspase-3 in these melanoma cell lines, there were marked differences between the cell lines in the breakdown of substrates for activated caspase-3. In cell lines that were sensitive to TRAIL, ICAD and PARP were degraded to a much greater extent than they were in the TRAIL-resistant melanoma lines. Moreover, the p17 subunit resulting from autocatalytic activity of caspase-3 was evident in the TRAIL-sensitive cell lines, but not in the TRAIL-resistant lines. The most likely explanation for these

[4] http://rsb.info.nih.gov/nih-image.

results was that activated caspase-3 was inhibited by one of the IAP family members, which was reported by others to bind to and inhibit the activity of caspase-3, as well as caspase-7 and caspase-9 (15, 16). This was supported by studies showing coimmunoprecipitation of XIAP and activated caspase-3. It was particularly noticeable that only the small subunit of caspase-3 (p20) was immunoprecipitated from the TRAIL-resistant cell line, whereas immunoprecipitates from the TRAIL-sensitive cell line also contained the p17 unit, which results when active caspase-3 cleaves its own prodomain (30, 31). Moreover, overexpression of XIAP in TRAIL-sensitive cell lines markedly suppressed TRAIL-mediated apoptosis.

To obtain further support for the role of XIAP as an inhibitor of caspase-3, we used two approaches to reduce its activity. One was to overexpress Smac/DIABLO in TRAIL-resistant melanoma cell lines because studies by others have shown that Smac/DIABLO specifically binds to and inactivates XIAP and other members of the IAP family (19, 20). These experiments showed that there was a marked increase in TRAIL-induced apoptosis in the transfected cell lines, and this was associated with an increase in autocatalytic activity by caspase-3 and breakdown of one of its substrates, PARP. The second approach was to deplete XIAP levels using the inhibitor of transcription Act-D or the protein synthesis inhibitor CHX (34). Pretreatment of TRAIL-resistant cell lines with these agents depleted XIAP (but not IAP1 and IAP2 levels or Bcl-2 and Bcl-X_L) and resulted in an increase of TRAIL-induced caspase-3 autocatalytic activity and apoptosis. These studies suggested that XIAP was mainly responsible for inhibition of caspase-3 but do not exclude the involvement of other IAP family members, particularly ML-IAP, which was not measured in the present study (35).

Although these studies clearly identified XIAP as a significant inhibitor of TRAIL-induced apoptosis, they did not explain the differences between cell lines that were sensitive to or resistant to TRAIL. This question was particularly relevant because Western blot studies did not show marked differences in the constitutive levels of XIAP (and IAP1 and IAP2) between the various melanoma cell lines. However, studies after exposure to TRAIL indicated that there was a marked decrease in XIAP levels in the TRAIL-sensitive cell lines, but not in the TRAIL-resistant cell lines. We examined whether this may be due to TRAIL-mediated release of Smac/DIABLO from mitochondria and found that TRAIL induced release of a much higher proportion of Smac/DIABLO from mitochondria of TRAIL-sensitive cells compared with that seen in TRAIL-resistant melanoma cells. Furthermore, the kinetics of Smac/DIABLO release was more consistent with the kinetics of down-regulation of XIAP, both of which were apparent by 30 min, whereas cytochrome *c* was not detectable until 2 h after the addition of TRAIL and was maximal at approximately 6 h. As expected, overexpression of Bcl-2 was associated with inhibition of Smac/DIABLO release, decreased autocatalytic activity of caspase-3, and TRAIL-mediated apoptosis.

These results are therefore consistent with the view that TRAIL mediates apoptosis of melanoma by release of Smac/DIABLO from mitochondria, which in turn blocks the XIAP-mediated inhibition of the effector caspase-3. The mechanism involved in reduction of XIAP levels may not be Smac/DIABLO itself but could be caspase-3 freed of the inhibitory effect of XIAP due to binding of Smac/DIABLO to XIAP (32, 33). It is conceivable that Smac/DIABLO release would increase the mitochondrial pathway to apoptosis by reducing XIAP-mediated inhibition of caspase-9. We believe that this explanation is less likely because of the kinetics of cytochrome *c* release discussed above and because the melanoma cells under study had high levels of activated caspase-3 induced by TRAIL. This would be unlikely to occur if there was significant inhibition of caspase-9 by XIAP or other IAP family members.

These studies indicate that TRAIL induces high levels of activated caspase-3 in approximately 36% of the melanoma cell lines in the present study. Whereas the results provide an explanation for the resistance of melanoma lines to TRAIL, they do not explain why TRAIL should induce a higher proportion of Smac/DIABLO release from mitochondria of some melanomas compared with others. This does not appear to be due to events upstream of the mitochondria, and closer examination of the role of the Bcl-2 family proteins is now needed to see whether their activity correlates with Smac/DIABLO release. It is also apparent that a variety of defects (other than inhibition by XIAP) exist in individual melanoma cell lines that make them resistant to TRAIL. This includes complete loss of receptors (5) and, as shown above for Me1007, loss of caspase-8. The mechanism of resistance of Mel-AT remains under study. Loss of caspase-8 was reported to be a common basis for resistance of neuroblastoma cells to TRAIL (36, 37).

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