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Bid and Calpains Cooperate to Trigger Oxaliplatin-Induced Apoptosis of Cervical Carcinoma HeLa Cells[S]

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ABSTRACT

The Bcl-2 homology 3-only protein Bid is an important mediator of death receptor-induced apoptosis. Recent reports and this study suggest that Bid may also mediate genotoxic drug-induced apoptosis of various human cancer cells. Here, we characterized the role of Bid and the mechanism of Bid activation during oxaliplatin-induced apoptosis of HeLa cervical cancer cells. Small hairpin RNA-mediated silencing of Bid protected HeLa cells against both death receptor- and oxaliplatin-induced apoptosis. Expression of a Bid mutant in which caspase-8 cleavage site was mutated (D59A) reactivated oxaliplatin-induced apoptosis in Bid-deficient cells but failed to reactivate death receptor-induced apoptosis, suggesting that caspase-8-mediated Bid cleavage did not contribute to oxaliplatin-induced apoptosis. Overexpression of *bcl-2* or treatment with the pan-caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone abolished caspase-2, -8, -9, and -3 activation as well as Bid cleavage in response to oxaliplatin, suggesting that Bid cleavage occurred downstream of mitochondrial permeabilization and was predominantly mediated by caspases. We also detected an early activation of calpains in response to oxaliplatin. Calpain inhibition reduced Bid cleavage, mitochondrial depolarization, and activation of caspase-9, -3, -2, and -8 in response to oxaliplatin. Further experiments, however, suggested that Bid cleavage by calpains was not a prerequisite for oxaliplatin-induced apoptosis: single-cell imaging experiments using a yellow fluorescent protein-Bid-cyan fluorescent protein probe demonstrated translocation of full-length Bid to mitochondria that was insensitive to calpain or caspase inhibition. Moreover, calpain inhibition showed a potent protective effect in Bid-silenced cells. In conclusion, our data suggest that calpains and Bid act in a cooperative, but mutually independent, manner to mediate oxaliplatin-induced apoptosis of HeLa cells.

BH3-only proteins are members of the Bcl-2 family of proteins with a key role in activation of apoptosis (Youle and Strasser, 2008). The BH3-only protein Bid has been well characterized in apoptosis triggered by ligands of the death receptors family (Kulik et al., 2001; Werner et al., 2002); Bid is cleaved by caspase-8 to t-Bid, which translocates to mitochondria and causes the release of caspase-activating factors from mitochondria (Korsmeyer et al., 2000; Seol et al., 2001). Bid can also be cleaved by other proteases, including granzymes (Waterhouse et al., 2006), cathepsins (Stoka et al., 2001; Reiners et al., 2002), and calpains (Chen et al., 2001; Mandic et al., 2002), events that potentially can also lead to the release of caspase-activating factors from mitochondria.

Oxaliplatin is a DNA-damaging agent belonging to a fam-

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ABBREVIATIONS: BH, Bcl-2 homology; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TNF, tumor necrosis factor; Ac-DEVD-AMC, *N*-acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin; zVAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp(*O*-methyl)-fluoromethylketone; tet, tetracycline; FRET fluorescence resonance energy transfer; FITC, fluorescein isothiocyanate; TMRM, tetra-methyl-rhodamine-methyl-ester; YFP, yellow fluorescent protein; CYP, cyan fluorescent protein; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP-ribose) polymerase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; STS, staurosporine; wt, wild type; MOMP, mitochondrial outer membrane permeabilization; BI-6C9, *N*-[4-[(4-aminophenyl)thio]phenyl]-4-[[(4-methoxyphenyl)sulfonyl]amino]-butanamide.

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ily of compounds that include cisplatin and carboplatin (Wang and Lippard, 2005); like cisplatin, oxaliplatin generates DNA inter- and intrastrand cross-links, but the spectrum of activity and the mechanisms of action and resistance are different from cisplatin and carboplatin (Cemazar et al., 2006; Kasparkova et al., 2008); the lack of cross-resistance with cisplatin and carboplatin (Rixe et al., 1996; Stordal et al., 2007) makes it a clinically important anticancer agent. Oxaliplatin-induced apoptosis of cancer cells has been shown to be partially dependent on the BH3-only protein PUMA (Wang et al., 2006; Köhler et al., 2008), which is activated in response to p53 during genotoxic stress. The involvement of Bid in DNA damage-induced apoptosis and genotoxic stress has been highlighted recently (Kamer et al., 2005; Zinkel et al., 2005, 2007; Kaufmann et al., 2007; Shelton et al., 2009), albeit with controversial findings. Although genetic ablation of Bid had no effects on apoptosis and cell cycle arrest in nontransformed murine cell types (Kaufmann et al., 2007), studies in human cancer cells have shown that Bid can mediate genotoxic stress-induced apoptosis and that Bid may act as a substrate for the genotoxic stress sensor ataxia-telangiectasia mutated kinase (Kamer et al., 2005; Zinkel et al., 2005). Bid was also shown to shuttle in and out of the nucleus to regulate cell cycle arrest (Zinkel et al., 2007). In a previous study from our laboratory, we were able to demonstrate that Bid was partially required and cooperated with PUMA in oxaliplatin-induced apoptosis of HeLa cells (Köhler et al., 2008). However, the mechanism of Bid activation and the potential role of other proteases in mediating Bid activation remained unanswered. In this study, we characterized the role of Bid and its mechanism of activation in oxaliplatin-induced apoptosis.

Materials and Methods

Materials. Human recombinant TRAIL was purchased from Leinco Technologies, Inc. (Universal Biologicals, Gloucestershire, UK). TNF-α was purchased from PeproTech (London, UK). Caspase substrate *N*-acteyl-Asp-Glu-Val-Asp-7-amino-4-methyl-coumarin (Ac-DEVD-AMC) and the pan-caspase inhibitor Z-Val-Ala-Asp(*O*-methyl)-fluoromethylketone (zVAD-fmk) were obtained from Bachem (St. Helen's, UK). All other drugs and chemicals came in analytical grade

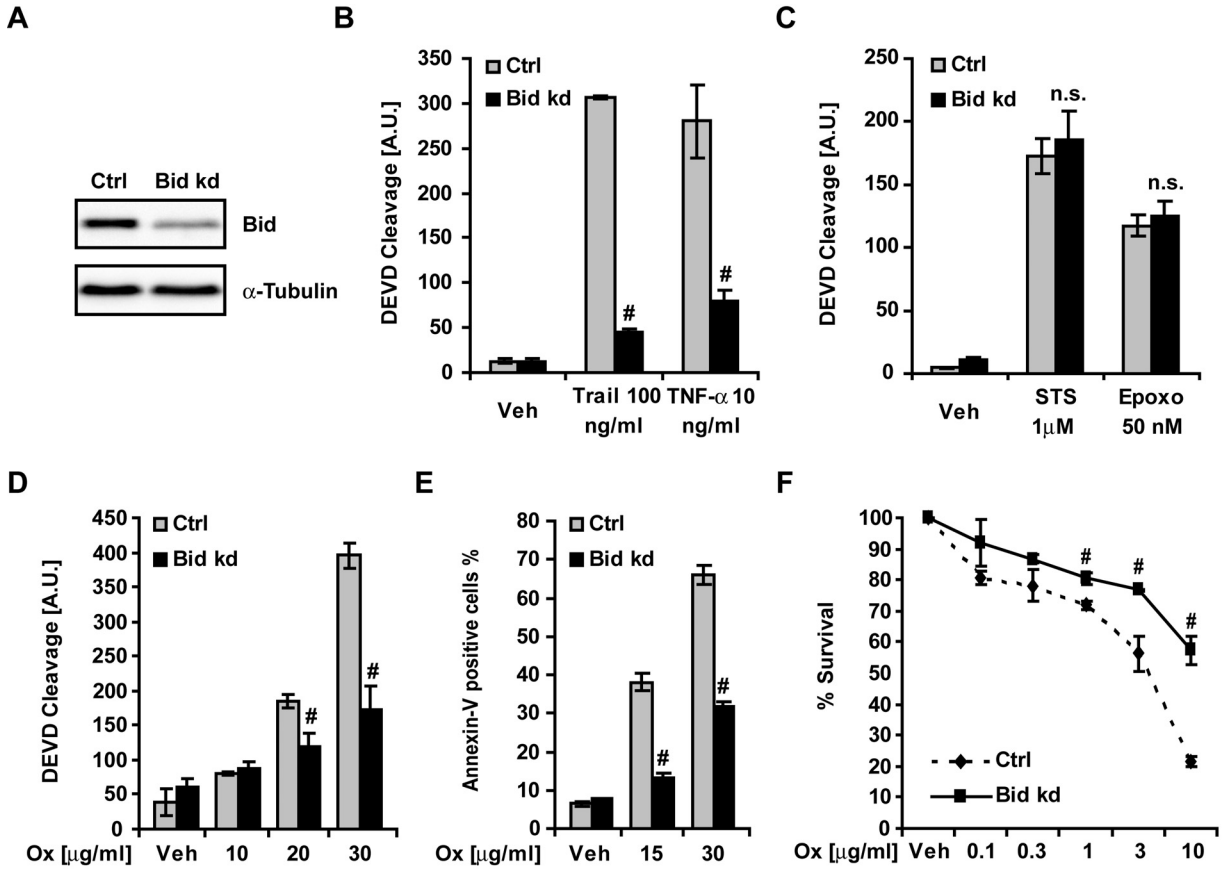

Fig. 1. Bid depletion inhibits death receptor-mediated apoptotic pathway and impairs oxaliplatin-induced cell death but does not affect staurosporine or proteasome inhibition-induced apoptosis. A, lysates from control and HeLa Bid kd were subjected to Western blotting with a polyclonal Bid antibody. β-Actin was used as loading control. B, control (Ctrl) and HeLa Bid kd cells were treated for the specified times with vehicle (Veh) or cyclohexamide (1 μg/ml) in combination with recombinant TRAIL or TNF-α. C and D, cells were treated with STS (6 h), epoxomicin (Epoxo; 24 h), or oxaliplatin (Ox; 24 h) at the indicated concentrations, or Veh. Caspase-3-like activity was measured by cleavage of the fluorogenic substrate Ac-DEVD-AMC. Data expressed as arbitrary units (A.U.) are means $\pm$ S.D. from $n = 3$ separate experiments. #, $p < 0.05$, significant difference from Ctrl cells. E, cells were treated as indicated, and apoptosis was assessed by flow cytometric evaluation of Annexin-V FITC-conjugated binding to phosphatidylserine in nonpermeabilized cells. Data are means $\pm$ S.D. from $n = 3$ separate experiments. #, $p < 0.05$, significant difference from Ctrl cells. F, control cells and HeLa Bid kd cells were treated with oxaliplatin at the indicated concentrations for 24 h. Controls were treated with vehicle. After incubation, 1000 cells were transferred to 60-mm dishes and cultured for 14 days, fixed, and counted. Graphical representation of the percentage of colonies after treatment compared with control cells treated with vehicle (100%). Data are means $\pm$ S.D. from at least two independent experiments performed in triplicate. #, $p < 0.05$, significantly different from Ctrl cells.

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Cell Culture. HeLa and HCT116 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma-Aldrich) in a humidified 5% CO_2 containing atmosphere at 37°C. Cells were kept in logarithmic growth phase by routinely passaging them twice a week, and they were plated 24 h before treatments. HeLa-DEVD cells have been described previously (Rehm et al., 2002). Bid knockdown-DEVD cells were generated using similar protocols.

Colony Formation Assay. Cells were seeded into 24-well plates and treated as indicated under Cell Culture. After 1 h of drug exposure, cells were resuspended with trypsin-EDTA; 1×10^3 cells were seeded onto 60-mm dishes and grown for 10 to 14 days to allow colonies to form. Medium was subsequently removed, and colonies were fixed/stained with a solution containing methanol (50%) and methylene blue (0.25%) at room temperature for 30 min. Plates were rinsed with water, and the number of colonies per plate was determined. Results are expressed as percentage of survival relative to the vehicle-treated controls (100%).

Expression of Adenoviral Bid wt and D59A Mutant. HeLa Bid kd cells were infected with adenoviral vectors coding for murine tetracycline-inducible FLAG-tagged wild-type or mutant (D59A) bid, kindly provided by Dr. A. Gross (Weizmann Institute of Science, Rehovot, Israel) (Sarig et al., 2003). Twenty-four hours before infection, 2×10^5 cells/well were seeded into six-well plates. After washing cells twice with serum-free medium, cells were infected with a multiplicity of infection of 1000 with the reverse tet transactivator-containing virus and either the wild-type or the mutant bid-containing virus. Fourteen hours after infection, doxycycline (1 μg/ml) was added to the medium to activate gene expression from the tet-inducible promoters. Recombinant bid protein expression was detectable after 6-h doxycycline treatment.

Flow Cytometric Analysis of Apoptosis and FRET. After apoptosis induction, HeLa or HCT116 cells were collected with trypsin-EDTA and incubated in binding buffer (10 mM HEPES, 135 mM NaCl, and 5 mM $CaCl_2$) containing Annexin-V FITC conjugated (5 μl/ml) (BioVision, Mountain View, CA) for 15-min shaking at 37°C. Then, 10^5 cells were resuspended in ice-cold binding buffer.

HeLa DEVD- or Bid-FRET cells were incubated with 100 nM tetra-methyl-rhodamine-methyl ester (TMRM) for 1 h at 37°C before sample preparation. Then, 10^5 cells were resuspended in ice-cold phosphate-buffered saline. Cells were subsequently analyzed on a CyFlow ML16 flow cytometer (Partec, Munster, Germany) equipped with a 200-mW argon ion laser emitting at 488 nm, a 100-mW diode laser emitting at 532 nm, and a 20-mW diode laser emitting at 405 nm.

Annexin-V was excited with 10% of the maximal intensity of the 488 nm laser, and fluorescence emission was collected in the FL1 channel through a band-pass filter at 515 to 555 nm; yellow fluorescence protein (YFP) was excited with 10% of the maximal intensity of the 488 nm laser, and fluorescence was collected through a band-pass filter at 515 to 555 nm; cyan fluorescence protein (CFP) was

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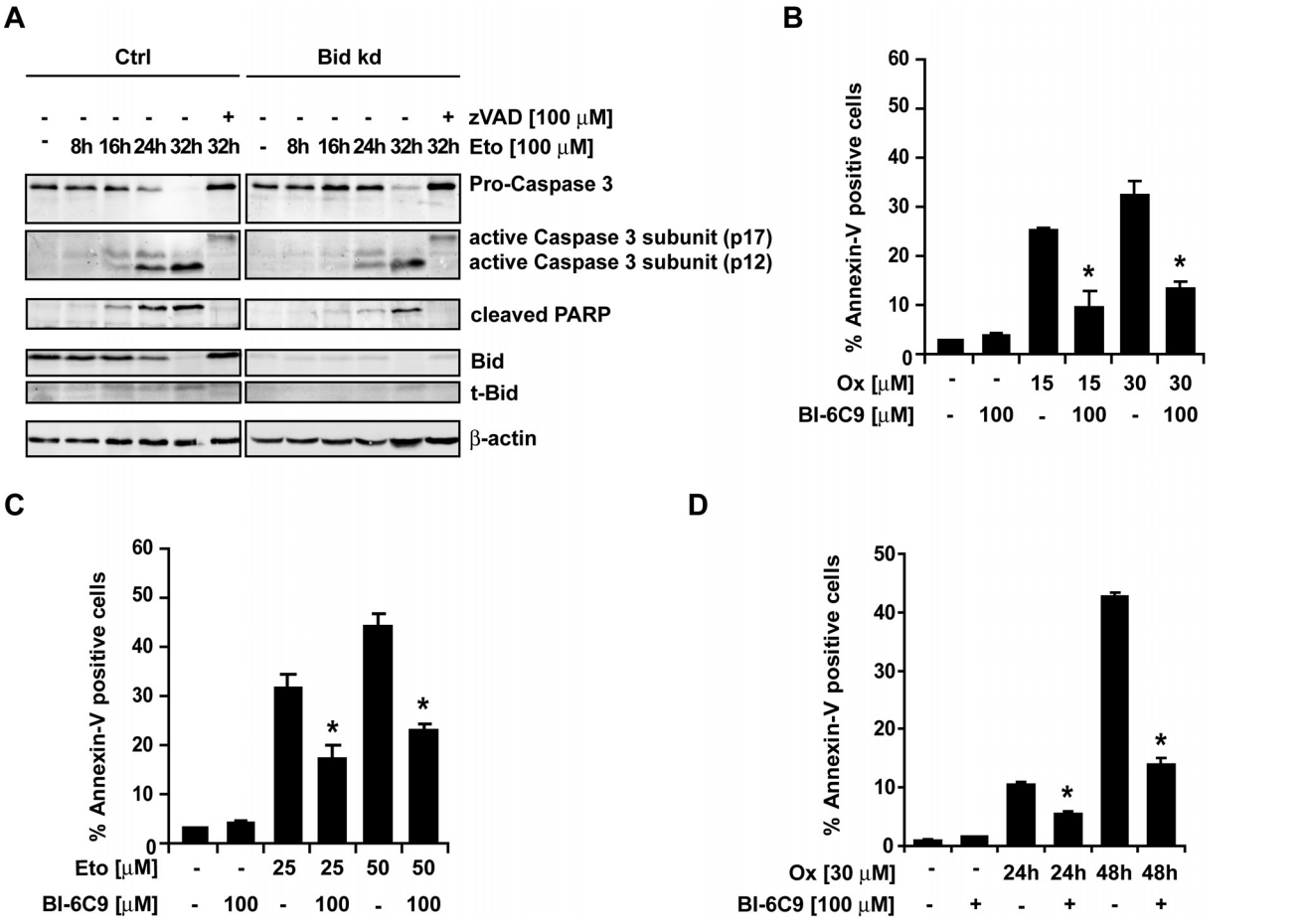

Fig. 2. Bid inhibition rescues cervical and colorectal cancer cells from oxaliplatin (Ox)- and etoposide (Eto)-induced apoptosis. A, control (Ctrl) and HeLa Bid kd cells were treated for the specified times with etoposide (100 μM) alone or in combination with the pan-caspase inhibitor zVAD-fmk. Lysates were subjected to Western blotting with a polyclonal caspase-3, a polyclonal cleaved PARP, a polyclonal Bid, and a monoclonal β-actin antibody. HeLa (B, C), and HCT116 (D) cells were treated with genotoxic drugs as indicated, and apoptosis was assessed by flow cytometric evaluation of Annexin-V FITC-conjugated binding to phosphatidylserine in nonpermeabilized cells. Data are means ± S.D. from $n = 3$ separate experiments. *, $p < 0.05$, significantly different from oxaliplatin-treated cells.

excited with the 405 nm laser, and fluorescence emission was collected through a band-pass filter at 435 to 475 nm; fluorescence resonance energy transfer (FRET) between CFP and YFP was collected through a band-pass filter at 515 to 555 nm. TMRM was excited with the 488 nm laser, and fluorescence emission was collected through a band-pass filter at 575 to 605 nm.

Data acquisition (10^4 gated events for each sample) and analysis were performed using FlowMax software (Partec). The order of magnitude of compensation parameters was 34% of YFP subtracted from TMRM, 4.5% of CFP subtracted from YFP, and 16% of CFP subtracted from FRET. The high degree of compensation between the YFP and the TMRM fluorescence signals is due to partial overlap in the emission spectrum. Data analysis was performed with FlowMax software. FRET fluorescent signals were shown as density plot diagrams. To point out the different cell populations, regions were selected on the following density plots: CFP versus YFP to exclude nonfluorescent cells, FRET versus CFP for cells with intact/cleaved probe, TMRM versus YFP for cells with normal or depolarized mitochondria, and FRET versus TMRM to isolate cells with depolarized mitochondria but intact caspase-3 FRET substrate.

Generation of Stable YFP-Bid-CFP Cells. HeLa D98 cells were transfected with 0.6 μg of plasmid DNA (pFRET-YFP-Bid-CFP) (Onuki et al., 2002) and 6 μl of Lipofectamine reagent (Invitrogen, Paisley, UK) per milliliter of serum-free culture medium at 37°C for 3 h. For the generation of stable cell lines, transfected HeLa D98 cells were selected in the presence of 1 mg/ml G418 (Invitrogen) for 2 weeks, and fluorescent clones were enriched. Expression of YFP-Bid-

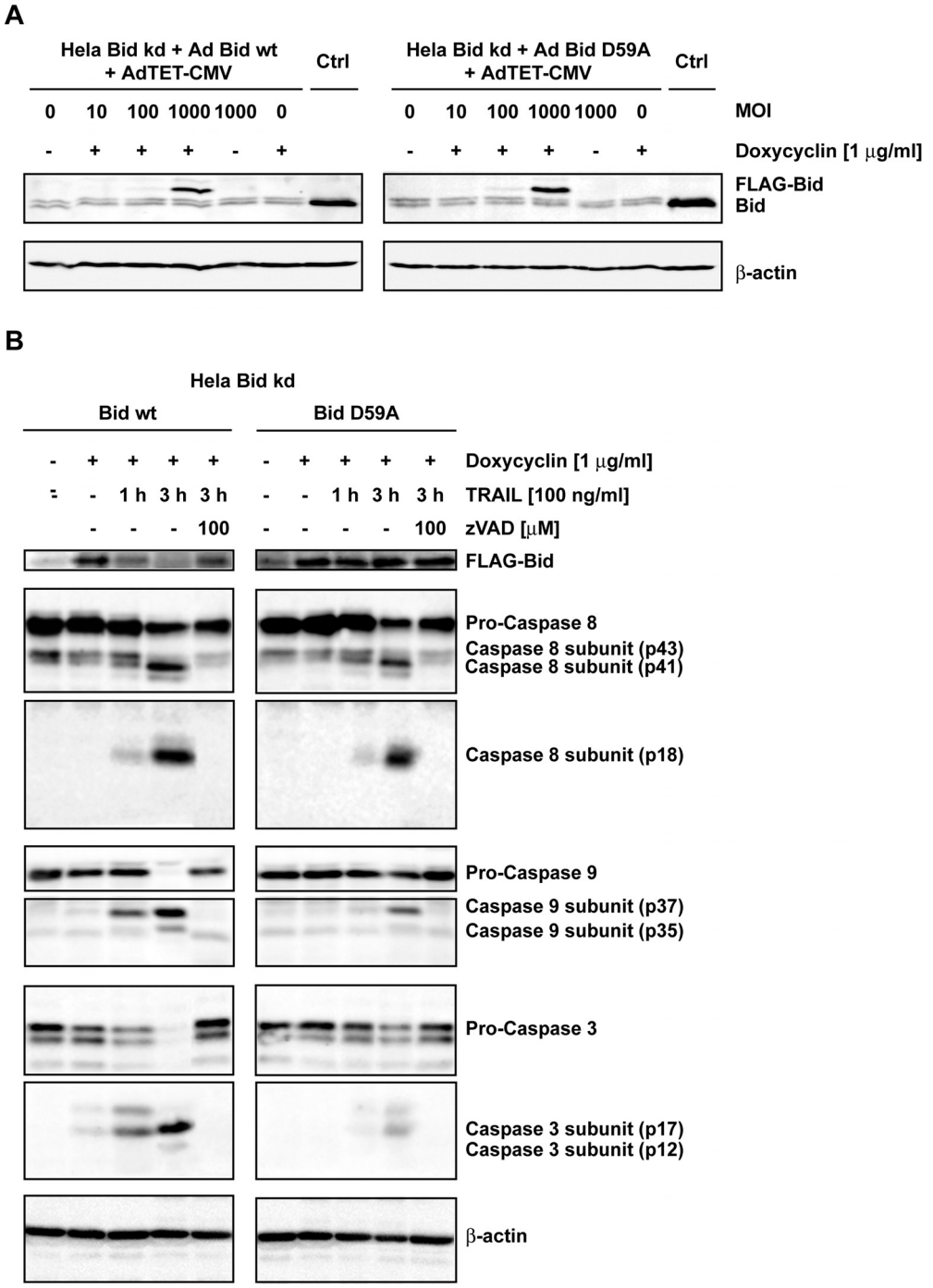

Fig. 3. Bid D59A mutant prevents caspase-9 and -3 activation downstream of caspase-8. A, HeLa Bid kd cells were infected with different concentrations of adenoviral particles encoding for tet-inducible wt Bid or Bid D59A. Twenty-four hours after infection, cells were treated with doxycycline (1 μg/ml) for 16 h, and then exogenous Bid expression was evaluated by Western blotting. Ctrl, control; MOI, multiplicity of infection. B, HeLa Bid kd cells infected with the tet-inducible Bid wt or bid D59A were preincubated with doxycycline (1 μg/ml) for 16 h and with the pan-caspase inhibitor zVAD-fmk (100 μM) for 1 h before treatment with cyclohexamide (1 μg/ml) in combination with recombinant TRAIL for the indicated times; lysates were subjected to Western blotting with a monoclonal caspase-8, a polyclonal caspase-9, a polyclonal caspase-3 antibody, and a monoclonal β-actin antibody.

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CFP was verified by immunoblotting using antibodies against GFP (data not shown).

SDS-PAGE and Western Blotting. Cells were rinsed with ice-cold phosphate-buffered saline and lysed in Tris-buffered saline containing SDS, glycerin, and protease inhibitors. Protein content was determined using the BCA Micro Protein Assay kit (Pierce Chemical, Rockford, IL). Samples were supplemented with 2-mercaptoethanol and denatured at 95°C for 5 min. An equal amount of protein (20–50 μg) was separated with 10% SDS-PAGE and blotted to nitrocellulose membranes (Protean BA 85; Schleicher & Schüll, Dassel, Germany). The blots were blocked with 5% nonfat milk in blocking solution (15 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 0.1% Tween 20) for 2 h at room temperature and incubated overnight with the primary antibody. The following primary antibodies were used: a mouse monoclonal Bcl-2 (Santa Cruz Biotechnology, Heidelberg, Germany), a goat polyclonal Bid (R&D Systems, Abingdon, UK), a mouse monoclonal calpain-4 (Millipore, Tullagreen, Ireland), a mouse monoclonal caspase-8 (Alexis Biochemicals, Blessington, Ireland), a rabbit polyclonal caspase-9 (Calbiochem, Darmstadt, Germany), a rabbit polyclonal caspase-3 (Cell Signaling Technology, Bray, Ireland), a mouse monoclonal caspase-2 (BD Biosciences Transduction Laboratories, Oxford, UK), a rabbit polyclonal GFP (Clontech, Oxford, UK), a rabbit polyclonal cleaved PARP (New England Biolabs, Bray, Ireland), and a mouse monoclonal β-actin or α-tubulin (Sigma-Aldrich). Horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., Cambridge, UK) were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Dublin, Ireland) and imaged using a FujiFilm LAS-3000 imaging system (Fuji, Tokyo, Japan).

Determination of Caspase-3-Like Protease Activity. Cells were lysed in 200 μl of lysis buffer (10 mM HEPES, pH 7.4, 42 mM KCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 5 μg/ml aprotinin, and 0.5% CHAPS). Fifty microliters of this lysate was added to 150 μl of reaction buffer (25 mM HEPES, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 3 mM dithiothreitol, pH 7.5, and 10 μM caspase substrate Ac-DEVD-AMC). Cleavage of the fluorogenic substrate Asp-Glu-Val-Asp-amino-4-methylcoumarin was monitored by measuring the accumulation of fluorescent 7-amino-4-methyl-coumarin after 1 and 2 h using a GENios microplate reader (Tecan, Crailsheim, Germany). Protein content was determined using the Coomassie Plus protein assay reagent (Pierce). Caspase activity was expressed as change in fluorescence per microgram of protein per hour.

Confocal Imaging Analysis of YFP-Bid-CFP Translocation and FRET Disruption. HeLa Bcl-2-overexpressing cells transiently transfected with the YFP-Bid-CFP FRET probe were cultivated in 35-mm glass-bottomed dishes (Wilcow BV, Amsterdam, The Netherlands) in 2 ml of medium overnight to let them attach firmly. Cells were treated with oxaliplatin (30 μg/ml) for 24 h and equilibrated with 30 nM TMRM in RPMI 1640 medium (Sigma-Aldrich) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal calf serum; buffered with 10 mM HEPES, pH 7.4; covered with mineral oil; and placed in a heated (37°C) incubation chamber that was mounted on the microscope stage. Images were collected through an LSM 510 meta confocal microscope (Carl Zeiss, Jena, Germany) equipped with a 488 nm argon ion laser, a 543 nm diode laser, and a 405 nm diode laser setup as described previously (Ward et al., 2006). FRET disruption was analyzed as described previously (Ward et al., 2006).

Statistics. Data are given as means ± S.D. or S.E.M. For statistical comparison, we used a *t* test or one-way analysis of variance followed by Tukey's test (SPSS software; SPSS GmbH Software, Munich, Germany). *p* values smaller than 0.05 were considered to be statistically significant.

Results

Bid Has a Central Role in Oxaliplatin-Induced Apoptosis. To analyze the contribution of Bid in apoptosis triggered by various proapoptotic stimuli, we used previously characterized HeLa cells with a stable knockdown of Bid, hereafter denoted HeLa Bid kd cells (Köhler et al., 2008) (Fig. 1A). HeLa Bid kd cells show no alterations in protein levels of other key apoptosis-regulating proteins (Köhler et al., 2008). As expected, detection of effector caspase activation by caspase activity assays indicated that the knockdown of Bid

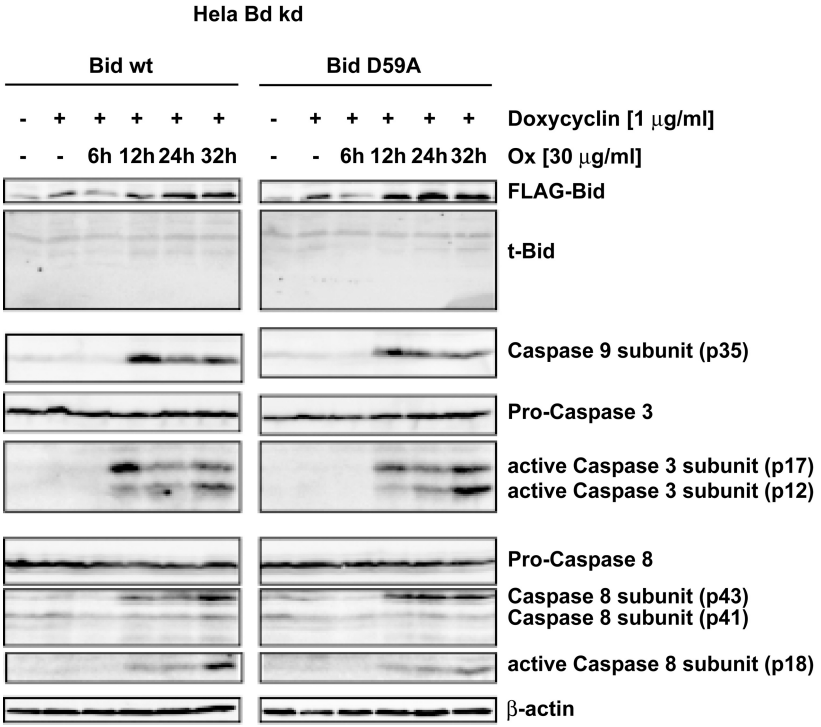

Fig. 4. Bid wt and Bid D59A induce comparable activation of the caspase cascade after oxaliplatin (Ox) treatment. HeLa Bid kd cells infected with the tet-inducible Bid wt or Bid D59A were preincubated with doxycycline (1 μg/ml) for 16 h before treatment with oxaliplatin for the indicated times; lysates were subjected to Western blotting with a monoclonal caspase-8, a polyclonal caspase-9, a polyclonal caspase-3 antibody, and a monoclonal β-actin antibody.

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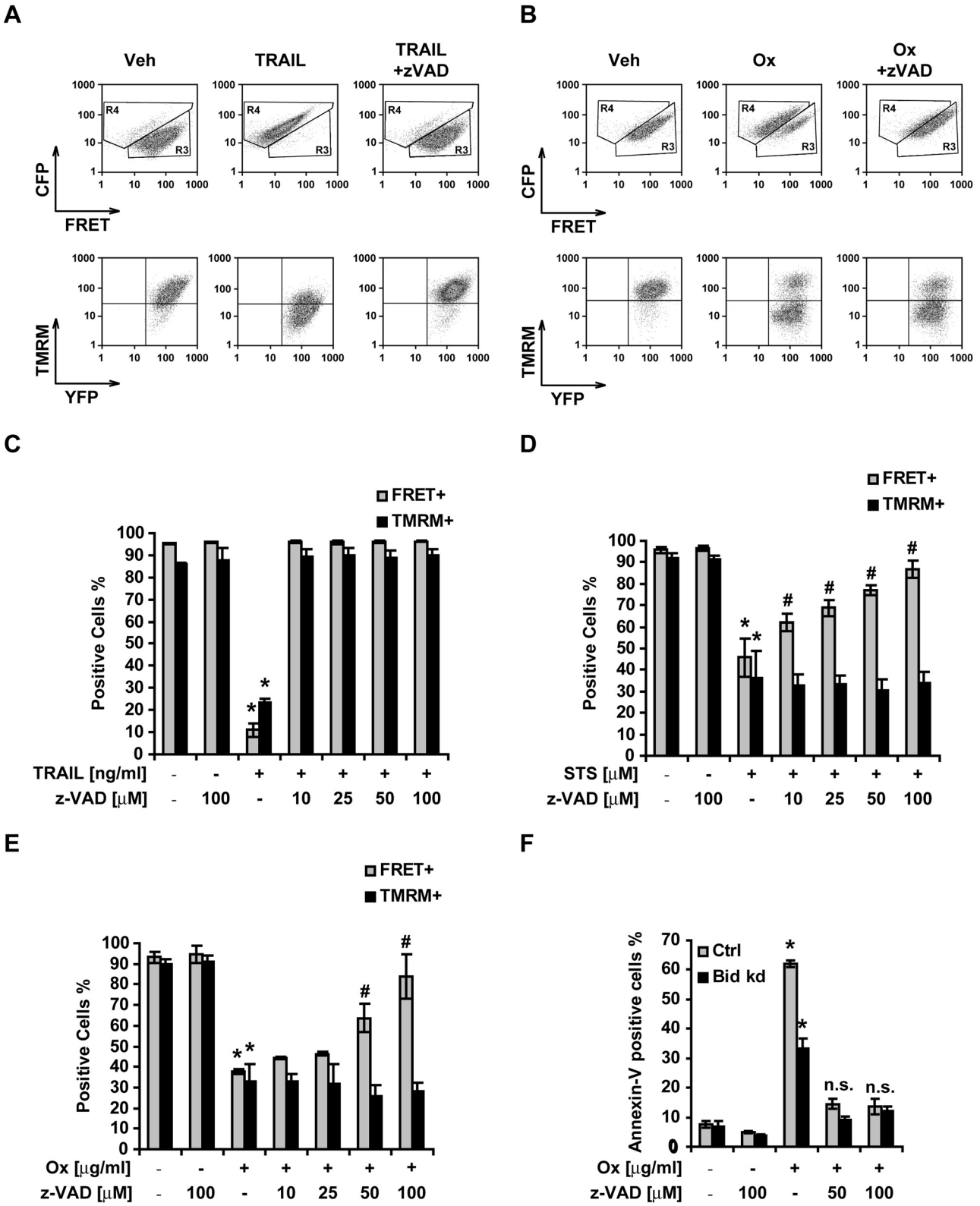

Fig. 5. Caspase inhibition prevents cleavage of a DEVD FRET probe and apoptosis in HeLa cells but does not prevent loss of mitochondrial membrane potential induced by oxaliplatin (Ox). HeLa DEVD cells were preincubated with the indicated concentrations of the pan-caspase inhibitor zVAD-fmk previous to treatment with recombinant TRAIL (100 ng/ml) for 4 h, STS (1 μM) for 6 h, and oxaliplatin (30 μg/ml) for 24 h. DEVD FRET probe cleavage and TMRM loss were evaluated by flow cytometry. Veh, vehicle. A and B, flow cytometry plots in representative samples are shown. Cell populations were gated to define FRET-positive cells (R3), FRET-negative cells (R4), TMRM-positive cells (top right quadrant), and TMRM-negative cells (bottom right quadrant). Veh, vehicle. C to E, quantification of the percentage of DEVD FRET probe cleavage and TMRM loss. Data are shown as percentage of FRET-positive cells (R4) and TMRM-positive cells (top right quadrant). Data are means ± S.D. from $n = 3$ separate experiments. *, $p < 0.05$, significant difference from control cells. #, $p < 0.05$, significant difference from treated cells. F, apoptosis in HeLa cells was assessed by flow cytometric evaluation of Annexin-V FITC-conjugated binding to phosphatidylserine in nonpermeabilized cells. Ctrl, control. Data are means ± S.D. from $n = 3$ separate experiments. *, $p < 0.05$, significant difference from Ctrl cells. n.s., not significant versus Ctrl.

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led to significantly impaired apoptosis triggered by the two death ligands TRAIL and TNF-α in HeLa cells (Fig. 1B). In contrast to death receptor-mediated apoptosis, the extent of staurosporine- (STS) or epoxomicin-induced effector caspase activation was not affected by the Bid knockdown (Fig. 1C). However, in agreement with our previous findings (Köhler et al., 2008), the knockdown of Bid significantly attenuated effector caspase activation, phosphatidylserine exposure, and clonogenic survival after induction of genotoxic stress with oxaliplatin (Fig. 1, D–F), indicating that Bid was involved in the proapoptotic pathways activated in response to this drug. HeLa Bid kd treated with etoposide also showed a reduced caspase-3 processing and PARP cleavage (Fig. 2A).

In a second attempt to demonstrate the involvement of Bid in oxaliplatin-induced cell death, we treated HeLa cells with the Bid inhibitor BI-6C9 (Becattini et al., 2006; Köhler et al., 2008). Treatment of HeLa cells with the Bid inhibitor significantly reduced the extent of apoptosis after oxaliplatin administration (Fig. 2B). The protection afforded by inhibition of Bid was also detected in cells exposed to other genotoxic drugs, including doxorubicin (Köhler et al., 2008) and etoposide (Fig. 2C). We then tested whether Bid inhibition also rescued other cancer cells from oxaliplatin-induced apoptosis. Treatment of HCT116 colon cancer cells with BI6C9 also led to a significant reduction in apoptosis in response to oxaliplatin (Fig. 2D).

Oxaliplatin-Induced Bid Activation Is Not Mediated by Caspase-8. To investigate the potential involvement of caspase-8 and -10 in oxaliplatin-mediated Bid activation, HeLa Bid kd cells were infected with an adenoviral vector inducibly expressing either FLAG-tagged wild-type Bid (Bid wt) or Bid D59A (Sarig et al., 2003) (Fig. 3A), a mutant in

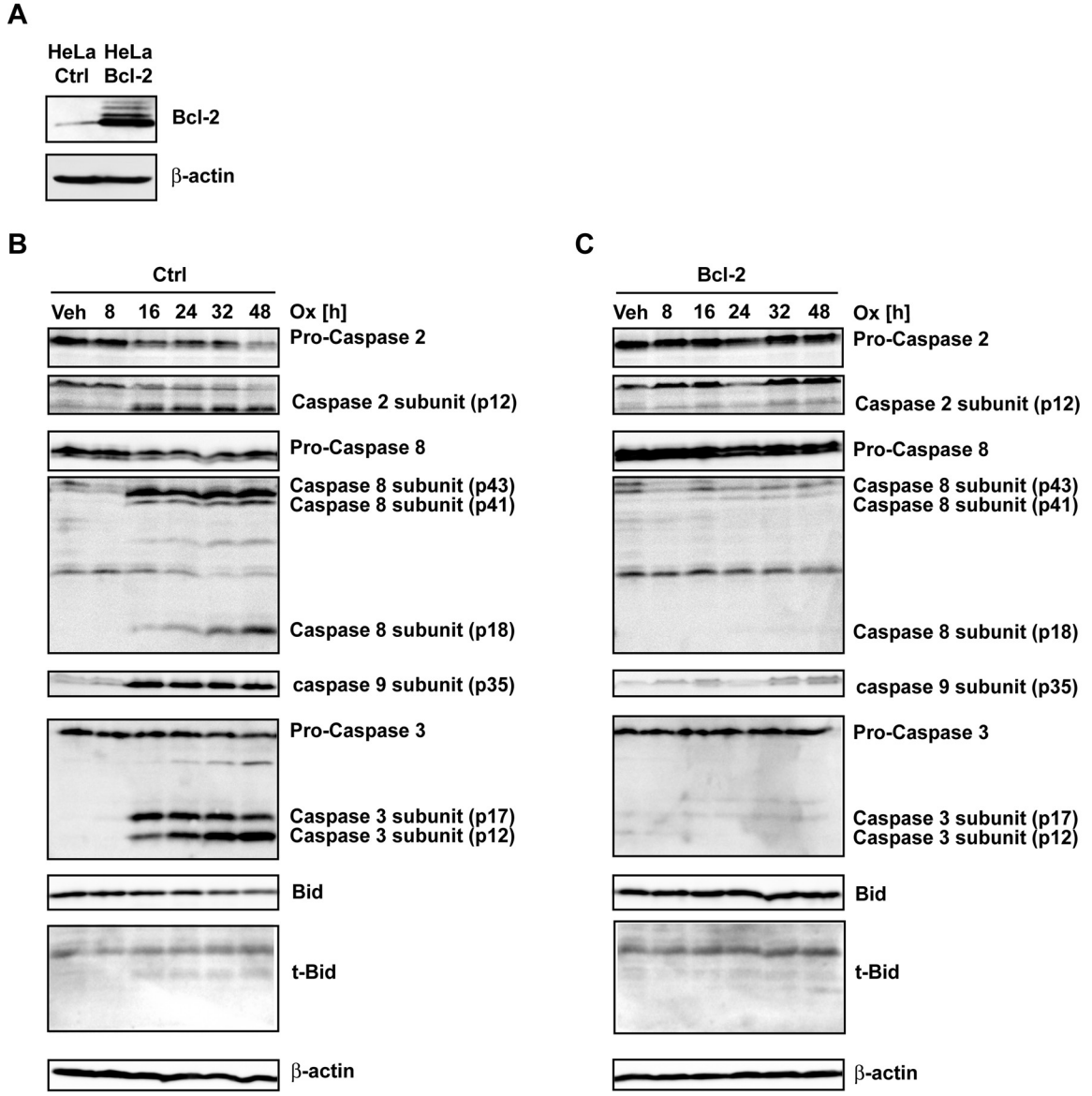

Fig. 6. Bcl-2 overexpression prevents processing of caspase-2, -3, -8, -9, and Bid cleavage in response to oxaliplatin (Ox). A, lysates from control and Bcl-2 overexpressing HeLa cells were subjected to Western blotting with a monoclonal Bcl-2 and a monoclonal β-actin antibody. Ctrl, control. B and C, control and Bcl-2-overexpressing HeLa cells were treated with oxaliplatin (30 μg/ml) for the indicated times; lysates were subjected to Western blotting with a monoclonal caspase-8, a polyclonal caspase-9, a polyclonal caspase-3, a monoclonal caspase-2, a polyclonal Bid, and a monoclonal β-actin antibody. Veh, vehicle.

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which the aspartic acid within the caspase cleavage motif at position 59 has been mutated to alanine, thus abolishing proteolytic cleavage by caspase-8 and -10. Western blot detection of Bid revealed that expression levels of adenovirally expressed Bid after viral transduction at 1000 multiplicity of infection in the presence of doxycycline in the Bid knockdown background were comparable with expression levels of endogenous Bid in HeLa control cells (Fig. 3A). Additional Western blot experiments did not detect differences in the kinetics of TRAIL-induced procaspase-8 activation in HeLa cells expressing either Bid wt or Bid D59A (Fig. 3B). After transduction with Bid wt, we observed cleavage of full-length Bid and processing of caspase-8, -9, and -3 to their active forms, all of which were inhibited by the pan-caspase inhibitor zVAD (Fig. 3B). The Bid D59A mutation abolished processing of Bid to t-Bid after TRAIL treatment, and this lack of proteolytic activation was associated with a pronounced decrease of caspase-9 and caspase-3 induction in the Bid knockdown background (Fig. 3B).

To analyze whether cleavage of Bid by caspase-8 or -10 was required for apoptosis induced by oxaliplatin, we performed a time course experiment (6–32 h) in which we analyzed the effects of the D59A mutation on oxaliplatin-induced activation of procaspase-8, -9, and -3 and on processing of Bid (Fig. 4). Our Western blots indicated that doxycycline increased protein levels of full-length Bid wt and Bid D59A in a time-dependent manner, whereas only minor levels of processed t-Bid could be observed in both cases, even at 32 h after oxaliplatin treatment (Fig. 4). It is interesting that the kinetics of Bid cleavage and activation of procaspase-9 and -3

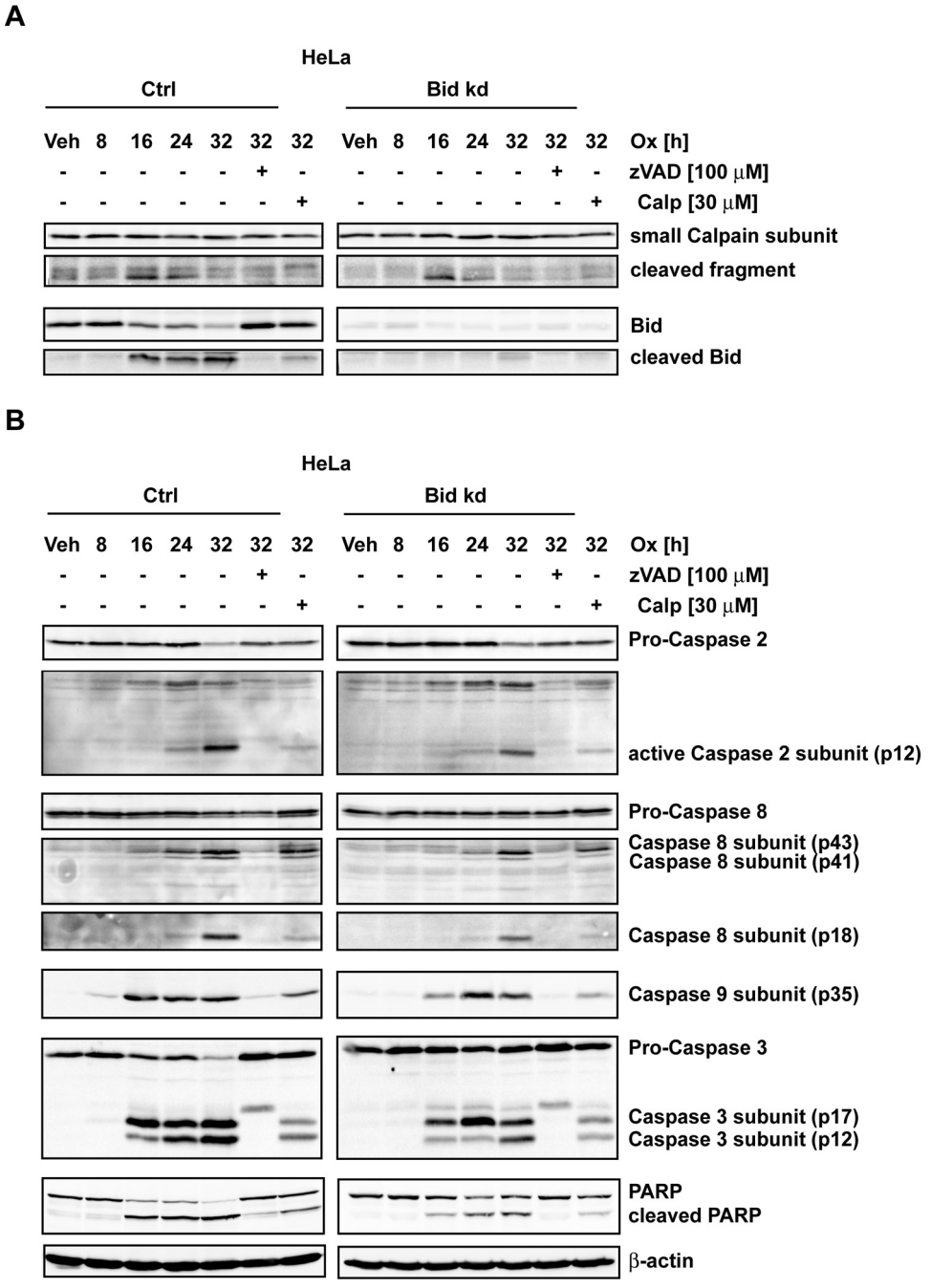

Fig. 7. Pharmacological inhibition of calpain protects against oxaliplatin (Ox)-induced apoptosis and further impairs activation of the caspase cascade in Bid kd cells. Control and HeLa Bid kd cells were treated for the specified times with oxaliplatin (20 μg/ml) alone or in combination with either the pan-caspase inhibitor zVAD-fmk or the calpain inhibitor calpeptin. A, lysates from control and Bid kd HeLa cells were subjected to Western blotting with a monoclonal calpain-4 antibody recognizing the autocatalytically cleaved 28-kDa small subunit shared by μ- and m-calpain, a polyclonal Bid, and a monoclonal β-actin antibody. B, lysates from control and Bid kd HeLa cells were subjected to Western blotting with a monoclonal caspase-2, a monoclonal caspase-8, a polyclonal caspase-9, a polyclonal caspase-3, a monoclonal PARP, and a monoclonal β-actin antibody. Veh, vehicle.

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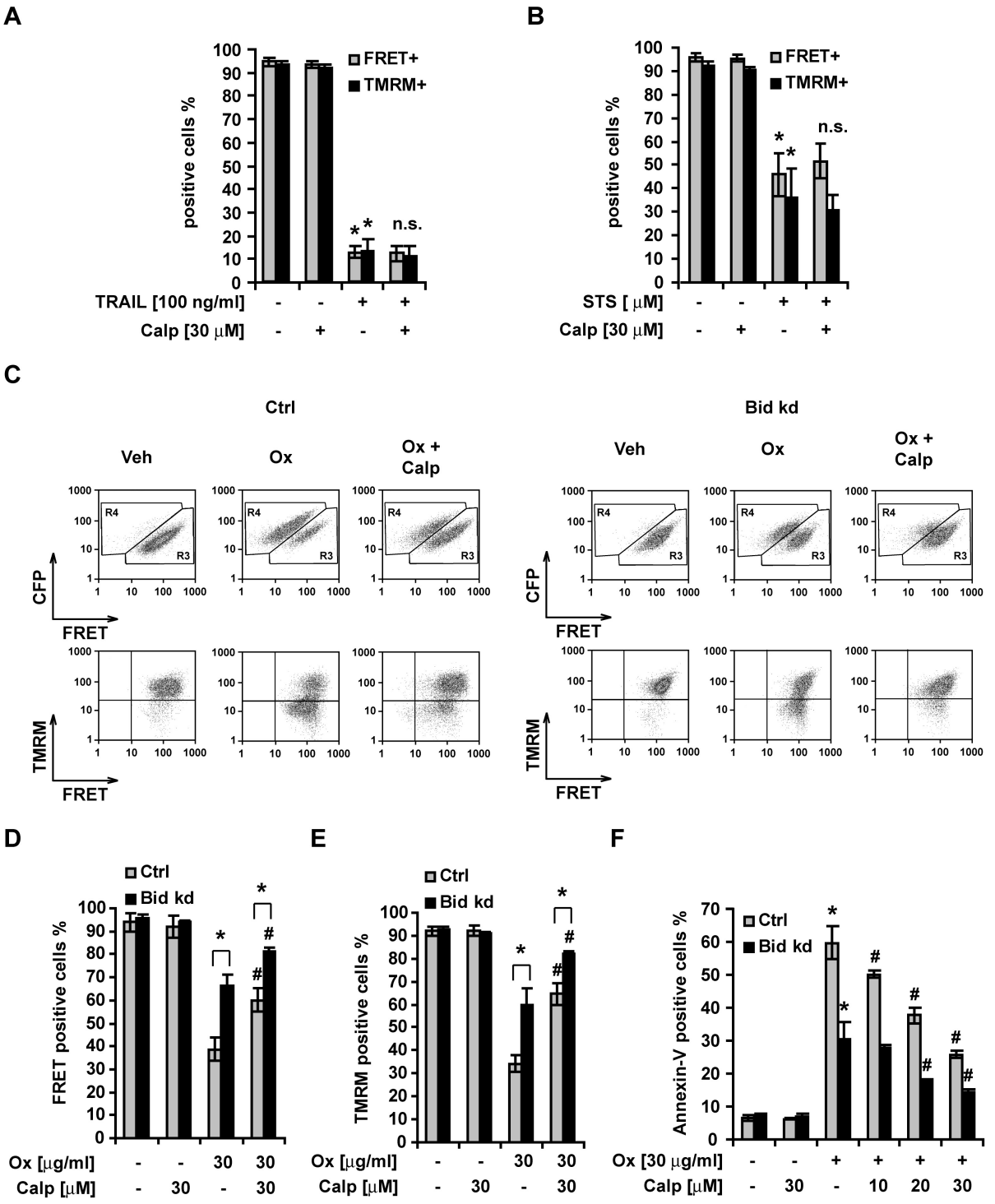

Fig. 8. Pharmacological inhibition of calpain recovers oxaliplatin (Ox)-induced DEVD FRET probe cleavage, mitochondrial membrane depolarization, and apoptosis. Stably transfected HeLa DEVD and HeLa Bid kd DEVD cells were preincubated with the indicated concentrations of the calpain inhibitor calpeptin (Calp) previous to treatment with TRAIL (100 ng/ml) for 4 h, STS (1 μM) for 6 h, oxaliplatin (30 μg/ml) for 24 h, or vehicle (Veh); DEVD FRET probe cleavage and TMRM loss were evaluated by flow cytometry. A and B, graphical representation of the percentage of DEVD FRET probe cleavage and TMRM loss. Data are shown as percentage of FRET-positive cells or TMRM-positive cells. Data are means ± S.D. from $n = 3$ separate experiments. *, $p < 0.05$, significant difference from control cells (Ctrl). n.s., nonsignificant from treated cells. C, flow cytometry plots in representative samples are shown. Cell populations were gated to define FRET-positive cells (R3), FRET-negative cells (R4), TMRM-positive cells (top right quadrant), and TMRM-negative cells (bottom right quadrant). Veh, vehicle. D and E, graphical representation of the percentage of DEVD FRET probe cleavage and TMRM loss. Data are shown as percentage of FRET-positive cells (R4) or TMRM-positive cells (top right quadrant), respectively. Data are means ± S.D. from $n = 3$ separate experiments. *, $p < 0.05$, significant difference from control (Ctrl) cells. #, $p < 0.05$, significant difference from oxaliplatin-treated cells. F, HeLa and HeLa Bid kd cells were treated with oxaliplatin (30 mg/ml) in combination with the calpain inhibitor calpeptin at the indicated concentrations for 24 h; apoptosis was measured by flow cytometric evaluation of Annexin-V binding to phosphatidylserine in nonpermeabilized cells. Data are means ± S.D. from $n = 3$ separate experiments. *, $p < 0.05$ difference from Ctrl cells. #, $p < 0.05$, significant difference from oxaliplatin-treated cells.

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were not affected by the Bid mutant, indicating that oxaliplatin-mediated activation of Bid and subsequent activation of the intrinsic pathway was independent of caspase-8 or -10.

Oxaliplatin-Induced Activation of Caspases Occurs Downstream of Mitochondria Engagement. To further investigate the extent of effector caspase activation after oxaliplatin treatment, control and HeLa Bid kd cells were stably transfected with a construct encoding a CFP-DEVD-YFP caspase-3/-7 substrate FRET probe (Rehm et al., 2002). We used flow cytometry techniques to simultaneously measure effector caspase activity and mitochondrial dysfunction by analyzing depolarization of mitochondrial membrane potentials with TMRM. To prevent potential postmitochondrial feedback mechanisms, we also used the pan-caspase inhibitor zVAD-fmk. In this experimental setting, effector caspase activation and mitochondrial depolarization induced by the death receptor ligand TRAIL were both inhibited by zVAD (Fig. 5, A and C), confirming that caspase activation occurs upstream of mitochondrial outer membrane permeabilization (MOMP) in death receptor-mediated apoptosis. In contrast, zVAD was able to uncouple mitochondrial dysfunction from effector caspase activation when the Bid-independent apoptotic stimulus STS was applied, because zVAD did not prevent mitochondrial depolarization in this case (Fig. 5D). These data indicated that STS-triggered caspase activation occurred downstream of MOMP. Surprisingly, similar experiments performed with oxaliplatin showed a profile comparable with that observed after treatment with STS (Fig. 5, B and E). Knockdown of Bid significantly reduced the percentage of cells displaying Annexin-V staining (Fig. 5F), depolarized mitochondria, and effector caspase activation (Supplemental Fig. 1). In a separate approach, we performed a Western blot analysis of caspase-2, -3, -8, and -9 processing after treatment with oxaliplatin in HeLa control cells versus HeLa cells overexpressing Bcl-2 (Fig. 6A). In control cells, all of these caspases were processed in the apoptotic cascade, albeit these experiments could not clearly resolve in what order they were activated (Fig. 6B). In the HeLa Bcl-2 cells, oxaliplatin-triggered activation of caspase-2 and -8 was abrogated, suggesting that both caspases require a caspase-3-dependent feedback loop for their activation in response to oxaliplatin. Partial cleavage of Bid to t-Bid, which was observed after oxaliplatin treatment of HeLa control cells (Fig. 6B), was completely blocked by overexpression of Bcl-2 (Fig. 6C).

Calpains Contribute to Oxaliplatin-Induced Apoptosis and Cooperate with Bid. Several families of proteases have been shown to cleave/activate Bid, including cathepsins (Blomgran et al., 2007), granzymes (Alimonti et al., 2001; Waterhouse et al., 2006), and calpains (Mandic et al., 2002). We could detect an early and significant activation of calpains in response to oxaliplatin treatment by Western blotting with an antibody recognizing the small subunit of μ-and m-calpain (calpain-4), which is autocatalytically cleaved after calpain activation. To assess whether calpains were involved in Bid activation after oxaliplatin treatment, we treated cells with the selective calpain inhibitor calpeptin. Western blot experiments detected reduced processing of m-, μ-calpain, and Bid cleavage (Fig. 7A) as well as reduced processing of caspase-2, -3, -8, and -9 (Fig. 7B) in the presence of calpeptin (Supplemental Fig. 1). The results demonstrated that calpain activation was involved in oxaliplatin-induced apoptosis and that calpains are activated upstream of mitochondria engagement.

These studies were further confirmed by flow cytometry studies. Calpeptin did not prevent mitochondrial membrane depolarization and effector caspase activation induced by TRAIL or STS as analyzed by flow cytometry, thereby confirming its specificity (Fig. 8, A and B); however, calpeptin significantly impaired effector caspase activation, mitochondrial dysfunction, and apoptosis after treatment of HeLa control cells with oxaliplatin (Fig. 8, C–F). To test the hypothesis whether the effects of calpain inhibition are mediated by Bid, we also studied the effect of calpeptin on apoptosis activation in HeLa Bid kd cells. It is interesting that the inhibitory effect of calpeptin was preserved in HeLa Bid kd cells (Fig. 8, C–F; Supplemental Fig. 1), suggesting a Bid-independent role for calpains in oxaliplatin-induced apoptosis. Similar to its effect in oxaliplatin-induced apoptosis, calpain inhibition also significantly reduced the activation of effector caspases in control and Bid-silenced HeLa cells exposed to etoposide (Fig. 9).

Full-Length Bid Translocates to the Mitochondria and Nucleus after Oxaliplatin Treatment, and Its Translocation Is Not Affected by Caspases or Calpain Inhibition. Although our data obtained so far had suggested that Bid plays an important role in oxaliplatin-induced apoptosis, we also had observed that Bid cleavage occurs largely downstream of mitochondria, suggesting that proteolytic ac-

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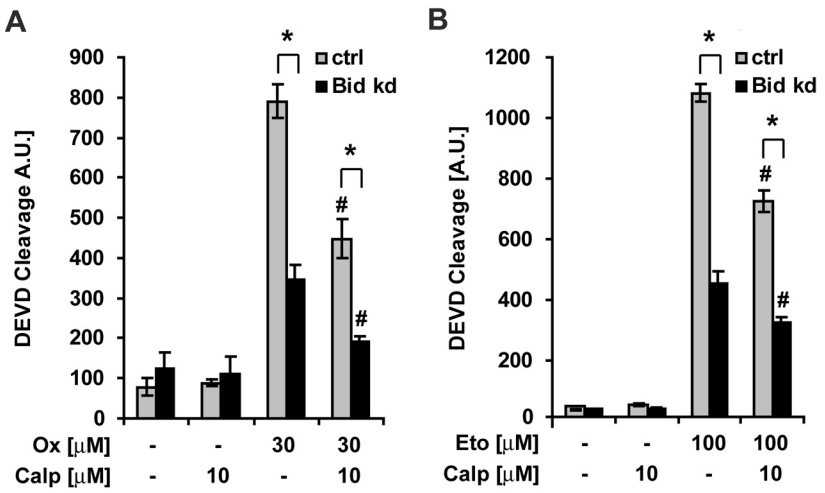

Fig. 9. Pharmacological inhibition of calpains (Calp) reduces caspase-3-like activity in oxaliplatin (Ox)- and etoposide (Eto)-treated HeLa control and Bid-depleted cells. Cells were treated with oxaliplatin (A) or etoposide (B) at the indicated concentrations for 24 h, or they received vehicle. Caspase-3-like activity was measured by cleavage of the fluorogenic substrate Ac-DEVD-AMC. Data are means ± S.D. from $n = 3$ separate experiments. *, $p < 0.05$, significant difference from control (ctrl) cells. #, $p < 0.05$, significant different from oxaliplatin-treated cells.

tivation of Bid may not be required for the proapoptotic function of Bid in this type of cell death. Previous studies have indicated that full-length Bid may also induce mitochondrial apoptosis in specific cell death settings (Sarig et al., 2003; Ward et al., 2006; König et al., 2007). To determine whether indeed full-length Bid has the capacity to translocate to mitochondria during oxaliplatin-induced apoptosis, a YFP-Bid-CFP FRET probe (Onuki et al., 2002; Ward et al., 2006) was transiently transfected into HeLa-Bcl-2 cells. In these cells, overexpression of Bcl-2 prevents MOMP, cytochrome *c* release, and subsequent effector caspase activation. This approach allowed for the detection of Bid activation in the absence of postmitochondrial positive feedback loops and with sufficient temporal and spatial resolution. Neither TRAIL nor oxaliplatin caused mitochondrial depolarization in Bcl-2-overexpressing cells (Fig. 10A), confirming the protective role of Bcl-2 in maintaining mitochondrial integrity. However, treatment with TRAIL was followed by cleavage of the YFP-Bid-CFP FRET probe, as analyzed by disruption of the FRET signal, and t-Bid-CFP, but not YFP-cBid, accumulated at mitochondria (Fig. 10A). In oxaliplatin-treated cells, the FRET signal remained intact, indicating that Bid was not

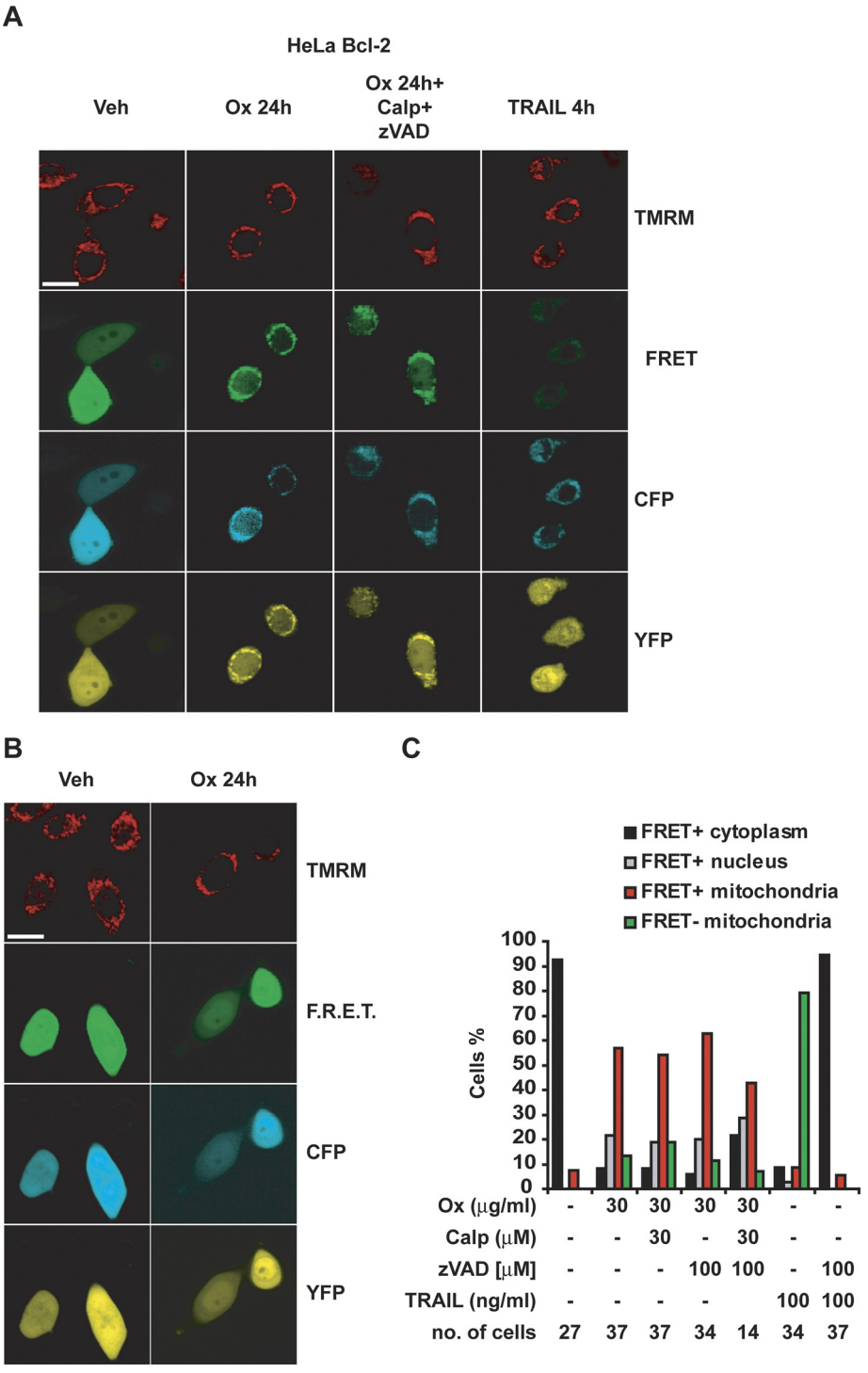

Fig. 10. Oxaliplatin (Ox)-induced full-length Bid translocation to mitochondria is not altered by either caspase or calpain inhibition. HeLa Bcl-2-overexpressing cells were transiently transfected with cDNA coding for the YFP-Bid-CFP fusion protein followed by treatment with oxaliplatin (30 μg/ml) or vehicle (Veh) for the indicated times. Where indicated, cells were preincubated with the pan-caspase inhibitor zVAD-fmk or the calpain inhibitor calpeptin (Calp). Cells were incubated with TMRM to visualize the mitochondria 1 h before collecting images. CFP, YFP, FRET, and TMRM fluorescence intensities were analyzed by confocal microscopy. A and B, confocal microscopy images of CFP, YFP, FRET, and TMRM fluorescence showing Bid cleavage and intracellular localization. Scale bar, 20 μm. C, quantitative analysis of full-length Bid intracellular localization. Bid intracellular distribution was analyzed by counting fluorescent cells displaying FRET signal at the different cellular compartments, cytoplasm, mitochondria, or nucleus.

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proteolytically processed. It is interesting that there was also a pronounced redistribution of the FRET, CFP, and YFP signals to the mitochondria, strongly suggesting that uncleaved, full-length YFP-Bid-CFP translocated to mitochondria after oxaliplatin treatment (Fig. 10A). Quantitative analysis confirmed that ~50% of the cells revealed a mitochondrial localization of YFP-Bid-CFP after treatment with oxaliplatin (Fig. 10C). There were no major changes in the number of cells displaying mitochondrial localization of full-length Bid when cells were cotreated with oxaliplatin in combination with calpeptin, zVAD, or calpeptin plus zVAD (Fig. 10C). Additional flow cytometry experiments of YFP-Bid-CFP cleavage in HeLa Bid kd cells supported the absence of Bid cleavage before mitochondrial depolarization in response to oxaliplatin (Supplemental Fig. 2). It is interesting that we also observed a significant translocation of CFP-Bid-YFP to the nuclear compartment during oxaliplatin-induced apoptosis (Fig. 10, B and C), as reported in previous studies (Kamer et al., 2005; Zinkel et al., 2005).

Discussion

In this study, we characterized the role of the BH3-only protein Bid and the mechanisms of Bid activation during apoptosis induced by the genotoxic anticancer drug oxaliplatin in HeLa cells. Our data demonstrate that silencing of Bid expression is associated with reduced apoptosis and increased clonogenic survival, thus underscoring the role of Bid in apoptosis triggered by oxaliplatin and other genotoxic drugs in human cancer cells. In addition, we provide evidence for a Bid-independent role of calpains in oxaliplatin- and etoposide-induced apoptosis.

Our study provides no evidence for Bid cleavage before mitochondrial dysfunction during oxaliplatin-induced apoptosis, although we are not able to fully exclude that a very small, nondetectable fraction of Bid may have been cleaved upstream of mitochondrial dysfunction. Canonical cleavage of Bid after death receptor activation is exerted via initiator caspase-8 or -10. In addition to caspase-8 and -10, caspase-2 has been described as an alternative, genotoxic stress-induced initiator caspase capable to catalyze Bid processing. In line with these observations, caspase-2 was activated by oxaliplatin in HeLa control cells. However, when activation of the intrinsic apoptosis pathway was blocked by overexpression of Bcl-2, oxaliplatin was not able to trigger a detectable cleavage of Bid, and it failed to activate caspase-2, as well as caspase-8, -9, and -3. In addition, treatment of HeLa cells with the pan-caspase inhibitor zVAD-fmk abolished activation of caspase-2, -8, -9, and -3 and the generation of t-Bid. These data suggested that although in oxaliplatin-treated HeLa cells caspase-2 and -8 may contribute to partial Bid cleavage, they were activated by a postmitochondrial feedback mechanism after subsequent activation of caspase-9 and -3. This lack of premitochondrial caspase-2 activation under conditions of genotoxic stress argues against an apical role of caspase-2 in genotoxic stress-triggered apoptosis in HeLa cells, as proposed for some other experimental models (Zhivotovsky and Orrenius, 2005; Manzl et al., 2009). Furthermore, treatment with zVAD did not delay or reduce the loss of mitochondrial membrane potentials after treatment with oxaliplatin. This also suggests that the postmitochondrial feedback loop encompassing caspase-3- and -6-dependent caspase-8 processing and Bid cleavage (Slee et al., 2000; Sohn et al., 2005) is not required for a full activation of the intrinsic pathway of apoptosis in HeLa cells. In line with these observations, further experiments with the Bid mutant D59A (Sarig et al., 2003) lacking the caspase cleavage site revealed that caspase-8 and -10 cleavage of Bid indeed did not significantly contribute to oxaliplatin-induced apoptosis, because the kinetics and extent of caspase-3 activation were not affected in comparison with cells expressing Bid wt. Collectively, these data suggest that Bid cleavage may represent a late event occurring at the postmitochondrial level and may be less important for initiation and execution of cell death.

Calpains are cytosolic cysteine proteases implicated in initiation and execution of cell death in many paradigms of apoptosis and caspase-independent cell death forms (Orrenius et al., 2003). Conventional m- and μ-calpains have been shown to be involved in regulation of apoptosis of human and murine cells after induction of genotoxic stress with cisplatin, camptothecin, etoposide, and ionizing radiation (Mandic et al., 2002; Tan et al., 2006; Liu et al., 2008). Mandic et al. (2002) reported that cisplatin-induced apoptosis of human melanoma cells involved calpain-dependent but caspase-independent Bid cleavage (Mandic et al., 2002). It is interesting that we also detected an early activation of calpains in response to oxaliplatin in HeLa cells. The synthetic and specific calpain inhibitor calpeptin was able to significantly block mitochondrial dysfunction and proteolytic activation of caspase-3, -2, -8, and -9 after treatment of HeLa control cells with oxaliplatin or etoposide. It is noteworthy that calpeptin also exerted potent antiapoptotic effects in Bid-deficient cells, suggesting that Bid and conventional calpains have additive, mutually independent proapoptotic roles in oxaliplatin- and etoposide-induced apoptosis. Active subunits of caspase-3, -2, -8, and -9 and autocatalytically generated cleavage fragments of calpains both were detectable in lysates of HeLa control cells and HeLa Bid kd cells after treatment with oxaliplatin for 16 h, but not at 8 h, allowing no further conclusions regarding the timely order of processing events in this signaling cascade. However, zVAD failed to prevent oxaliplatin-induced mitochondrial depolarization, demonstrating that calpains could promote mitochondrial dysfunction in the absence of caspase activation. Furthermore, the significantly reduced activation of caspase-3, -2, -8, and -9 in the presence of calpain inhibitors suggests that calpains are positioned upstream of caspase activation in this type of cell death.

Our data obtained so far had suggested that Bid plays an essential role but that its cleavage is dispensable for oxaliplatin-induced apoptosis. We and others have demonstrated previously that Bid can translocate to the mitochondria in its full-length form and can trigger the activation of the intrinsic apoptosis pathway (Sarig et al., 2003; Ward et al., 2006; König et al., 2007). Single-cell imaging experiments in Bcl-2-overexpressing HeLa cells using a YFP-Bid-CFP FRET probe indeed demonstrated an accumulation of full-length Bid at mitochondria in response to oxaliplatin that was not attenuated by the presence of zVAD and/or calpeptin (Fig. 10). Although the mitochondrion-specific lipid cardiolipin is known to act as the mitochondrial receptor of t-Bid (Lutter et al., 2000), mitochondrial targeting of full-length Bid may depend on other mechanisms, such as interaction with phosphatidic acid and phosphatidylglycerol (Esposti et al., 2001)

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or interaction with phosphofurin acidic cluster sorting protein-2 (Simmen et al., 2005). It is interesting that inhibition of phosphatidylcholine synthesis and accumulation of phosphatidic acid and phosphatidylglycerol are hallmarks of cell death pathways characterized by Ca^{2+} signaling (Gasull et al., 2003; König et al., 2007). It remains to be shown whether the accumulation of full-length Bid at "primed" mitochondria is sufficient to trigger a direct activation of Bax during oxaliplatin-induced apoptosis or whether the effects of Bid are mediated via alternative covalent modifications. It is therefore possible that specific stress signals such as those induced by oxaliplatin trigger changes in the intracellular phospholipid environment that enable full-length Bid to translocate to mitochondria and interact with other Bcl-2 family proteins.

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