Dephosphorylation Targets Bcl-2 for Ubiquitin-dependent Degradation: A Link between the Apoptosome and the Proteasome Pathway

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Summary

Injury of the endothelial cells by the induction of apoptotic cell death may play an important role in the pathophysiology of atherosclerosis and the progression of inflammatory diseases. Here, we demonstrate an essential role for the ubiquitin-dependent proteasome complex in stimulus-induced degradation of the antiapoptotic protein Bcl-2. Bcl-2 is specifically degraded after stimulation of human endothelial cells with tumor necrosis factor (TNF)-α in a process that is inhibited by specific proteasome inhibitors. In addition, the mutation of the potential ubiquitin-acceptor amino acids of Bcl-2 provides protection against TNF-α– and staurosporine-induced degradation in vitro and in vivo. Moreover, mimicking phosphorylation of the putative mitogen-activated protein (MAP) kinase sites of the Bcl-2 protein (Thr 56, Thr 74, and Ser 87) abolishes its degradation, suggesting a link between the MAP kinase pathway to the proteasome pathway. Finally, inhibition of Bcl-2 degradation either by suppressing ubiquitin-dependent proteasomal degradation or by mimicking continuous phosphorylation of the putative MAP kinase sites in the Bcl-2 protein confers resistance against induction of apoptosis. Thus, the degradation of Bcl-2 may unleash the inhibitory function of Bcl-2 over the apoptosome and may thereby amplify the activation of the caspase cascade.

Key words: Bcl-2 • apoptosis • mitogen-activated protein kinase • endothelial cells • proteasome

Apoptosis, the programmed kind of cell death, is an important physiological process in the development and homeostasis of multicellular organisms. Apoptotic cell death is characterized by a common pattern of morphological alterations such as nuclear condensation, membrane blebbing, and DNA fragmentation. The derangement of apoptosis either by depression of this suicidal program or by an overstimulation may be linked to pathophysiological disorders such as cancer or neurodegenerative diseases (1). Importantly, injury of the endothelial monolayer by the induction of apoptotic cell death may play an important role in the pathophysiology of atherosclerosis and the progression of inflammatory diseases (2–6).

The signals leading to apoptotic cell death have been mainly discovered in the nematode *Caenorhabditis elegans*, but are highly conserved among species, with at least three classes of proteins playing important roles encoded by the C. *elegans* cell death (Ced)[1] gene, ced-3, ced-4, and ced-9 (7, 8). The mammalian homologues of the ced-3 gene are cysteine proteases with aspartic acid specificity (caspases). Caspases are the key effector proteins of apoptosis in mammalian cells (9). The mammalian homologues of *ced-9* have been identified and comprise proteins of the Bcl-2 family. Bcl-2, Mcl-1, and Bcl-X_L have been shown to promote cell survival, whereas other members of the Bcl-2 family, such as Bax, Bad, or Bcl-X_S, exhibit proapoptotic effects (10, 11). The mammalian homologue of Ced-4 has recently been identified as Apaf-1 (12). Two distinctly different pathways of caspase activation and apoptosis have been delineated (13). First, ligation of death receptors such as Fas recruits adaptor proteins and procaspase molecules like caspase-8, resulting in direct activation of the caspase cascade (14). In the second pathway, various forms of cellular stress trigger mitochondrial release of cytochrome C, which binds to Apaf-1, leading to the activation of downstream caspases (15, 16). Thus, although both pathways converge on the activation of the downstream caspases, the involvement of cytochrome C released from mitochondria introduces a fundamental difference. Importantly, Bcl-2 has been shown to prevent cytochrome C release from mito-

[1]*Abbreviations used in this paper:* ALLN, *N*-acetyl-leucinyl-leucinyl-norleucinal-H; Ced, *Caenorhabditis elegans* cell death gene; ERK, extracellular signal–regulated kinase; HUVEC, human umbilical vein endothelial cell; MAP, mitogen-activated protein; MKP, mitogen-activated protein kinase phosphatase; Z-LLL-H, carbobenzoxyl-leucinyl-leucinyl-leucinal-H.

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chondria (17, 18). Thus, the model currently proposed to account for the antiapoptotic action of Bcl-2 is that Bcl-2 interferes with activation of the cytochrome C/Apaf-1 pathway by stabilizing the mitochondrial membrane. Inflammatory processes or heart failure are associated with a dramatic decrease of Bcl-2 protein levels in vivo (19–21), which correlates with in vitro studies demonstrating a posttranscriptional reduction of Bcl-2 (22, 23). Thus, posttranscriptional regulation of Bcl-2 may significantly affect the resistance of cells to apoptosis induction.

The degradation of intracellular proteins is mainly mediated by the ubiquitin-dependent proteasome complex (24). Thereby, proteins are targeted for degradation by the covalent attachment of ubiquitin, a ubiquitously expressed 76–amino acid polypeptide (25, 26). Initially, the degradation of proteins by the proteasome complex was regarded as a mechanism for destruction of misfolded or damaged proteins. However, it is now clear that proteasome-mediated degradation plays a crucial role in regulating various essential cell functions (27). Indeed, the ubiquitin-dependent proteasome pathway controls the ordered degradation of proteins involved in cell cycle control, and further regulates cell survival by degradation of p53 (28, 29). Moreover, several other important signaling pathways, such as the activation of transcription factors, are controlled via protein degradation by the proteasome complex (30, 31).

In this study, we examined the role of Bcl-2 degradation in apoptosis signaling. We demonstrated that Bcl-2 is specifically degraded in human umbilical vein endothelial cells (HUVECs) or HeLa cells undergoing stimulus-dependent apoptosis. The enzyme responsible for Bcl-2 degradation was identified as the ubiquitin-dependent proteasome complex, and caspases were not involved. Characterization of the signaling events triggering Bcl-2 degradation indicates a link between the mitogen-activated protein (MAP) kinase pathway and the proteasome pathway. Most important, we provide evidence that inhibition of Bcl-2 degradation either by suppressing ubiquitin-dependent proteasomal degradation or by mimicking continuous phosphorylation of MAP kinase sites in the Bcl-2 protein confers protection against TNF-α– or staurosporine-mediated apoptosis.

Materials and Methods

Cell Culture. HUVECs were purchased from Cell Systems/Clonetics and cultured in endothelial basal medium supplemented with hydrocortisone (1 μg/ml), bovine brain extract (3 μg/ml), gentamicin (50 μg/ml), amphotericin B (50 μg/ml), epidermal growth factor (10 μg/ml), and 10% FCS until the third passage.

Western Blot Analysis and Immunoprecipitation. HUVECs (5×10^5 cells) were incubated for the time indicated with TNF-α, and homogenates were obtained as described previously (3); Western blots were performed with anti–Bcl-2 antibody (Boehringer Mannheim) or anti-myc antibody (Santa Cruz Biotechnology). For detection of phosphorylated extracellular signal–regulated kinase (ERK), proteins were isolated as described previously (32), and blots were probed with phospho-specific ERK1/2 (New England Biolabs). For immunoprecipitation, proteins (3 mg) were incubated with anti-myc antibody (1 μg/ml; Santa Cruz Biotechnology) at 4°C for 4 h and bound to A/G-PLUS–agarose beads (Santa Cruz Biotechnology). Western blot analysis was performed using antiubiquitin antibody (Sigma Chemical Co.).

Plasmid Transfection. Human Bcl-2 was amplified by PCR with oligonucleotides containing EcoRV and HindIII restriction sites and cloned into the respective sites of pcDNA3.1(−)Myc-His (Invitrogen) under the transcriptional control of the CMV promoter. MAP kinase phosphatase (MKP)-3 was cloned after PCR amplification with oligonucleotides containing EcoRV and BamHI restriction sites into the pcDNA3.1(−)Myc-His vector. Bcl-2 mutants and the MKP-3 mutant were obtained by PCR-directed mutagenesis or by site-directed mutagenesis (Stratagene). Clones with verified sequences were transfected in HeLa cells or HUVECs. HeLa cells were transfected with plasmids encoding wild-type Bcl-2 or mutated Bcl-2 by the lipofectamine procedure (GIBCO BRL) and selected with 0.5 mg/ml G-418 for 6 d before stimulation of apoptosis. Heterogenous populations of the stably transfected cells were used to avoid any possible clonal variations. HUVECs were transfected with 3 μg pcDNA3.1 plasmid encoding the corresponding sequence as described previously (32). For the detection of apoptosis, HUVECs were cotransfected with β-galactosidase. In brief, 150 μl medium was mixed with 3 μg plasmids (1 μg pcDNA3.1-lacZ and 2 μg pcDNA3.1–Bcl-2) and 30 μl Superfect (Qiagen) and incubated for 10 min at room temperature. During the incubation time, medium was removed from the cell culture plates, and HUVECs were washed twice in medium without FCS. 1 ml medium was then added to the plasmid-Superfect mixture, and HUVECs were incubated with this mixture for 3 h at 37°C. After the incubation, culture medium was removed, 3 ml fresh complete medium was added, and HUVECs were incubated for 36 h to allow protein expression. Transfected cells were identified by β-galactosidase staining (32). Viable versus dead stained cells were counted by two blinded investigators, and results were expressed as (dead cells/viable cells) ×100. In addition, necrotic cell death was excluded by measuring lactate dehydrogenase (LDH) release, thus indicating that the cell death of the transfected cells is caused by apoptosis. The transfection efficiency with 3 μg pcDNA3.1–β-galactosidase was 24 ± 4%.

Pulse–Chase Analysis. HUVECs were starved in RPMI medium without methionine and cysteine for 1 h, then metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine for 3 h. HUVECs were then chased in nonradioactive medium for the time periods indicated, in the presence or absence of TNF-α. Cells were washed twice with PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, and 50 mM Tris-HCl, pH 8) at 4°C for 20 min. Samples containing equal amounts of protein were precleared with anti–rabbit IgG for 30 min at 4°C, immunoprecipitated with an anti-myc antibody, and resolved on 12% SDS-PAGE. The gel was dried and exposed to x-ray film.

In Vitro Translation. pcDNA3.1–Bcl-2 wild-type or mutants were in vitro transcribed/translated using the T7 polymerase kit (Promega Corp.) in the presence of [^{35}S]methionine. Degradation was then determined by incubation of ^{35}S-labeled Bcl-2 with TNF-α–treated HUVEC homogenates (70 μg) at 37°C.

Cell Death Analysis. DNA fragmentation was demonstrated by the typical DNA laddering and quantified by an ELISA specific for histone-associated DNA fragments as described previously (3). For morphological staining of nuclei, cells were centrifuged (10 min, 700 *g*), fixed in 4% formaldehyde, and stained with 4′,6-diamidino-phenylindole (DAPI; 0.2 μg/ml in

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10 mM Tris-HCl, pH 7, 10 mM EDTA, 100 mM NaCl) for 20 min.

Phosphorylation. For detection of in vitro phosphorylation of Bcl-2 by MAP kinase, COS cells were transfected with myc-tagged Bcl-2 wild-type or mutant, and overexpressed proteins were immunoprecipitated with anti-myc antibodies. The immunoprecipitates were incubated at 30°C in 30 μl kinase reaction mixture containing 25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 2 mM dithiothreitol, 10 mM $MgCl_2$, 50 μM ATP, 5 μCi [γ-^{32}P]ATP with or without 20 ng activated MAP kinase ERK2 (New England Biolabs) for 30 min. The reaction was terminated by addition of SDS loading dye, and samples were subjected to a 12% SDS-PAGE and analyzed by PhosphorImager (Molecular Dynamics).

Results and Discussion

Selective Degradation of Bcl-2 by Apoptotic Stimuli. Treatment of HUVECs with the proapoptotic stimuli TNF-α, staurosporine, and doxorubicin resulted in a profound time- and dose-dependent reduction of Bcl-2 protein levels (Fig. 1, A and B; data not shown). Reduction of Bcl-2 protein levels clearly preceded the induction of apoptosis (Fig. 1 B). In contrast, TNF-α did not affect Bax or Bcl-X_L protein levels (Fig. 1 C). Reduced Bcl-2 protein levels in response to various apoptotic stimuli were also observed in HeLa cells, illustrating that the effects are not unique for endothelial cells (data not shown). Bcl-2 mRNA levels

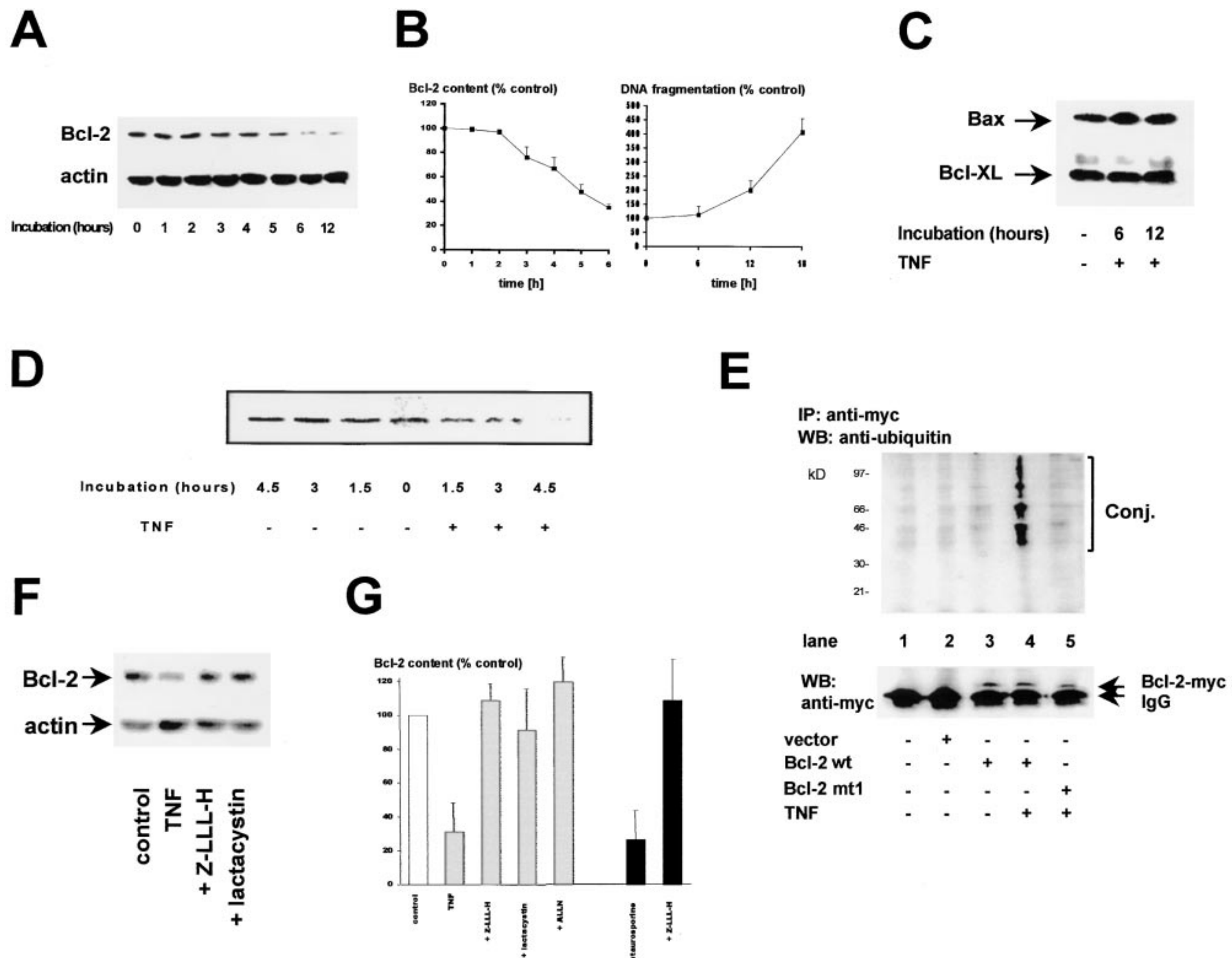

Figure 1. TNF-α–induced Bcl-2 degradation. (A) Time-dependent reduction of Bcl-2 in TNF-α–stimulated HUVECs. Western blot analysis of Bcl-2 protein after incubation of HUVECs with TNF-α (100 ng/ml) for the time periods indicated. Blots were reprobed with actin as a loading control. (B) Effect of TNF-α on Bcl-2 protein levels and apoptosis. Bcl-2 protein levels were determined by densitometric analysis of Bcl-2 Western blots after incubation of HUVECs with TNF-α for the time periods indicated. Apoptosis was measured by an ELISA specific for histone-associated DNA fragments ($n = 5$; mean ± SD). (C) Western blot analysis for Bax and Bcl-X_L after incubation of HUVECs with TNF-α (100 ng/ml) for 6 or 12 h. (D) Pulse–chase experiment. HUVECs were transiently transfected with myc-tagged Bcl-2 for 24 h, then chased for 30 min before incubation for the indicated times in the presence or absence of TNF-α, and Bcl-2 was immunoprecipitated with an anti-myc antibody. (E) Formation of Bcl-2 ubiquitin adducts. HUVECs were transfected with myc-tagged wild-type (wt) Bcl-2 or the Bcl-2 mutant (mt) lacking all lysine residues (K17R, K22R, K218R, and K239R). After stimulation with TNF-α for 2.5 h, the expressed Bcl-2 protein was immunoprecipitated with anti-myc antibodies, and ubiquitination was detected by Western blotting (WB) with antiubiquitin antibodies. Reprobe of the Western blot with antibodies against myc (lower panel) serves as control for expression and immunoprecipitation. (F and G) Effect of the proteasome inhibitors Z-LLL-H (20 μM), lactacystin (25 μM), and ALLN (5 μg/ml) on TNF-α–induced (100 ng/ml, 6 h) or staurosporine-induced (1 μM, 6 h) Bcl-2 degradation ($n = 4$; mean ± SD).

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were essentially unchanged after TNF-α–induced apoptosis (data not shown), suggesting a posttranscriptional effect on Bcl-2 protein. Pulse–chase experiments demonstrated that an increased rate of Bcl-2 protein degradation rather than a reduced protein biosynthesis accounts for the decline of Bcl-2 protein levels (Fig. 1 D). Thus, TNF-α, staurosporine, and doxorubicin appear to stimulate the selective degradation of Bcl-2 by a posttranscriptional mechanism.

The Ubiquitin-dependent Proteasome Pathway Mediates Bcl-2 Degradation. The ubiquitin-dependent proteasome pathway plays an important role in the degradation of regulatory proteins and thereby fulfills important functions in cell cycle regulation and signal transduction (30, 31). The ubiquitin-dependent pathway requires the covalent conjugation of ubiquitin with ϵ-amino groups of lysine residues within the target proteins (24). Indeed, TNF-α stimulated the ubiquitination of transiently expressed myc-tagged Bcl-2 (Fig. 1 E, lane 4). In contrast, ubiquitination was significantly prevented in a Bcl-2 construct, where all four lysine residues were mutated to arginine (Fig. 1 E, lane 5). Moreover, the proteasome inhibitors lactacystin (33, 34), carbobenzoxyl-leucinyl-leucinyl-leucinal-H (Z-LLL-H [31]), and *N*-acetyl-leucinyl-leucinyl-norleucinal-H (ALLN [31]) completely prevented TNF-α– as well as staurosporine-induced degradation of Bcl-2 in vivo (Fig. 1, F and G).

Similar results were obtained in an in vitro assay system in which the cleavage of ^{35}S-labeled Bcl-2 by homogenates of TNF-α–treated HUVECs was assessed (Fig. 2 A). The proteolytic cleavage of ^{35}S-labeled Bcl-2 was abolished by the specific proteasome complex inhibitors (Fig. 2 B). More-

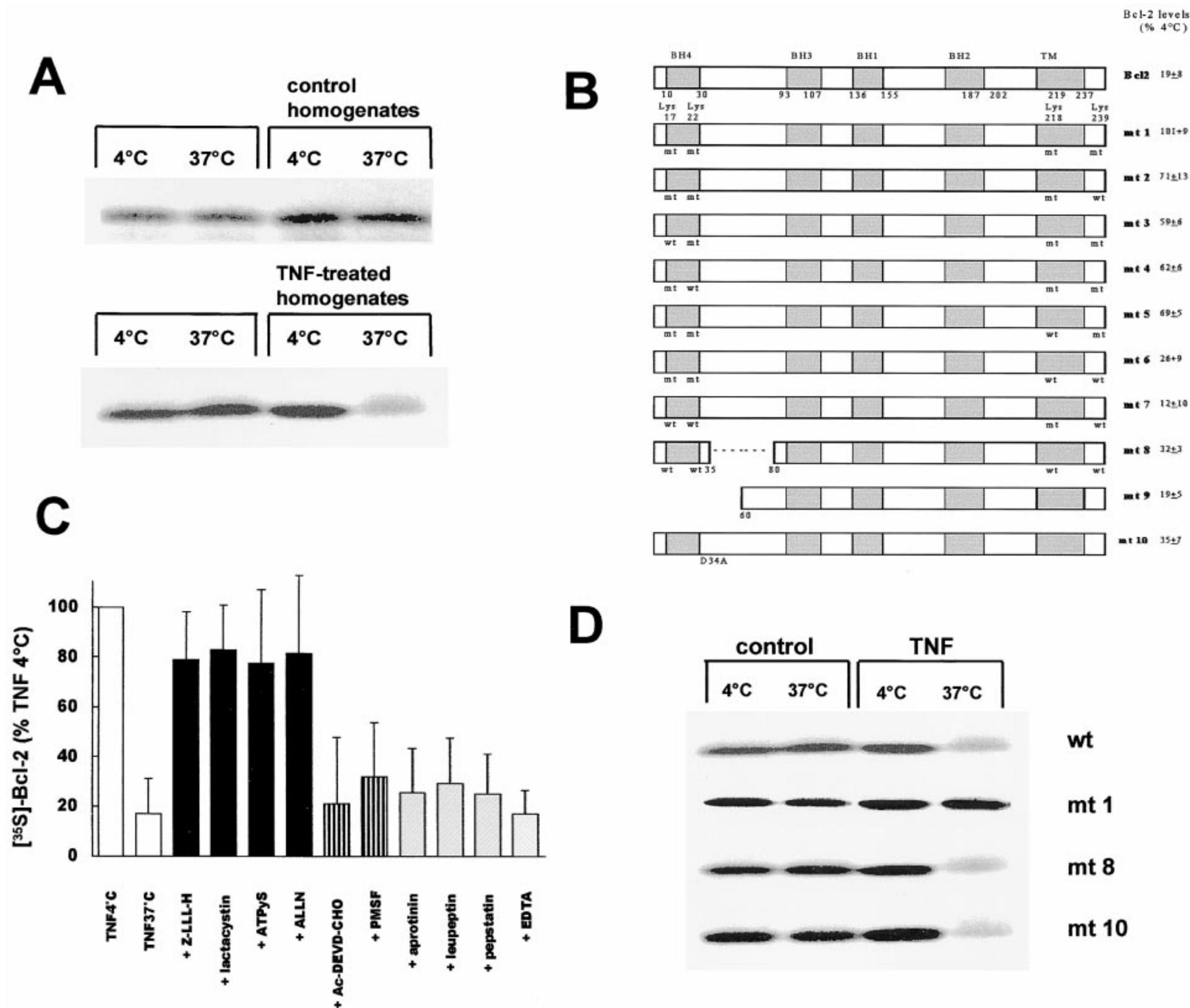

Figure 2. Characterization of TNF-α–induced Bcl-2 degradation in vitro. (A) Comparison of Bcl-2 cleavage activity of HUVEC homogenates, which were prestimulated for 2.5 h with or without TNF-α. ^{35}S-labeled Bcl-2 was coincubated in the presence or absence of HUVEC homogenates for 3 h at 4 or 37°C. (B) Inhibition of TNF-α–stimulated ^{35}S–Bcl-2 cleavage activity in HUVEC homogenates by specific proteasome inhibitors (Z-LLL-H, 40 μM; lactacystin, 50 μM; ALLN, 5 μg/ml), caspase-3 inhibitor (Ac-DEVD-CHO, 100 μM), serine protease inhibitors (PMSF, 5 mM; aprotinin, 1 μg/ml; leupeptin, 1 μg/ml), aspartic protease inhibitor (pepstatin, 1 μg/ml), or metalloprotease inhibitor (EDTA, 10 mM) or ATPγS (2 mM). Densitometric data are expressed as mean ± SEM (n = 4). (C) Illustration of mutants and reduction of Bcl-2 levels after incubation with TNF-α–prestimulated homogenates at 37°C compared with 4°C (mean ± SEM; n = 3). Mutated (mt) and wild-type (wt) lysine residue are indicated. (D) Autoradiographs demonstrating the proteolytic cleavage of selected mutants induced by TNF-α–prestimulated HUVEC homogenates, when incubated for 3 h at 4 or 37°C.

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over, the degradation of Bcl-2 was inhibited by ATPγS (Fig. 2 B), which is a typical feature of the proteasome complex (35). Furthermore, mutating all four lysine residues (mt 1) precluding ubiquitination as shown in Fig. 1 E completely abrogated the degradation of Bcl-2 (Fig. 2, C and D). Mutation of three out of four lysine residues (mt 2–5) led to a partial inhibition of Bcl-2 degradation (Fig. 2, C and D). In contrast, caspase inhibitors (Ac-DEVD-CHO, Ac-YVAD-CHO) or inhibitors of serine-, aspartate-, or metalloproteases did not prevent Bcl-2 degradation (Fig. 2 B; data not shown). Mutation of the caspase cleavage site Asp^{34} of Bcl-2 (36) did not confer resistance against TNF-α–mediated Bcl-2 degradation in vitro or in vivo (mt 10, Fig. 2, C and D; data not shown). Additionally, mutation of the loop region (amino acid residues 35–79) of Bcl-2 did not affect stimulus-dependent degradation (mt 8, Fig. 2, C and D). These results indicate that the ubiquitin-dependent proteasome pathway is required for TNF-α–induced degradation of Bcl-2.

Inhibition of the Ubiquitin-dependent Proteasome Pathway Confers Resistance to Proapoptotic Stimuli. To establish a functional role of stimulus-dependent degradation of Bcl-2 on apoptosis induction, the degradation-resistant Bcl-2 mutant (mt 1) was transfected into HUVECs. TNF-α–induced apoptosis was significantly reduced in cells expressing the corresponding Bcl-2 mutant, whereas overexpression of wild-type Bcl-2 provided less protection (Fig. 3, A and B). Likewise, expressing the degradation-resistant Bcl-2 mutant in HeLa cells significantly reduced TNF-α–induced apoptosis by 71 ± 6% in Bcl-2 mutant transfected cells compared with 41 ± 0.7% reduction in wild-type Bcl-2 transfected cells ($P < 0.05$), indicating that the observed effects are not unique to endothelial cells. In contrast, overexpression of the caspase cleavage–resistant D34A Bcl-2 mutant did not prevent TNF-α–induced apoptosis in HUVECs (19 ± 3 compared with 20.7 ± 4% apoptosis in Bcl-2 wild-type transfected cells). Moreover, apoptosis induced by staurosporine was dramatically inhibited by overexpression of the degradation-resistant Bcl-2 mutant, which rescued almost all cells (Fig. 3 C). The different effects of the Bcl-2 mutants were not due to enhanced basal expression as demonstrated by Western blot analysis (Fig. 3, B and C). Thus, these results establish inhibition of the ubiquitin-dependent degradation of Bcl-2 as an extremely potent mechanism to suppress stimulus-induced apoptosis induction.

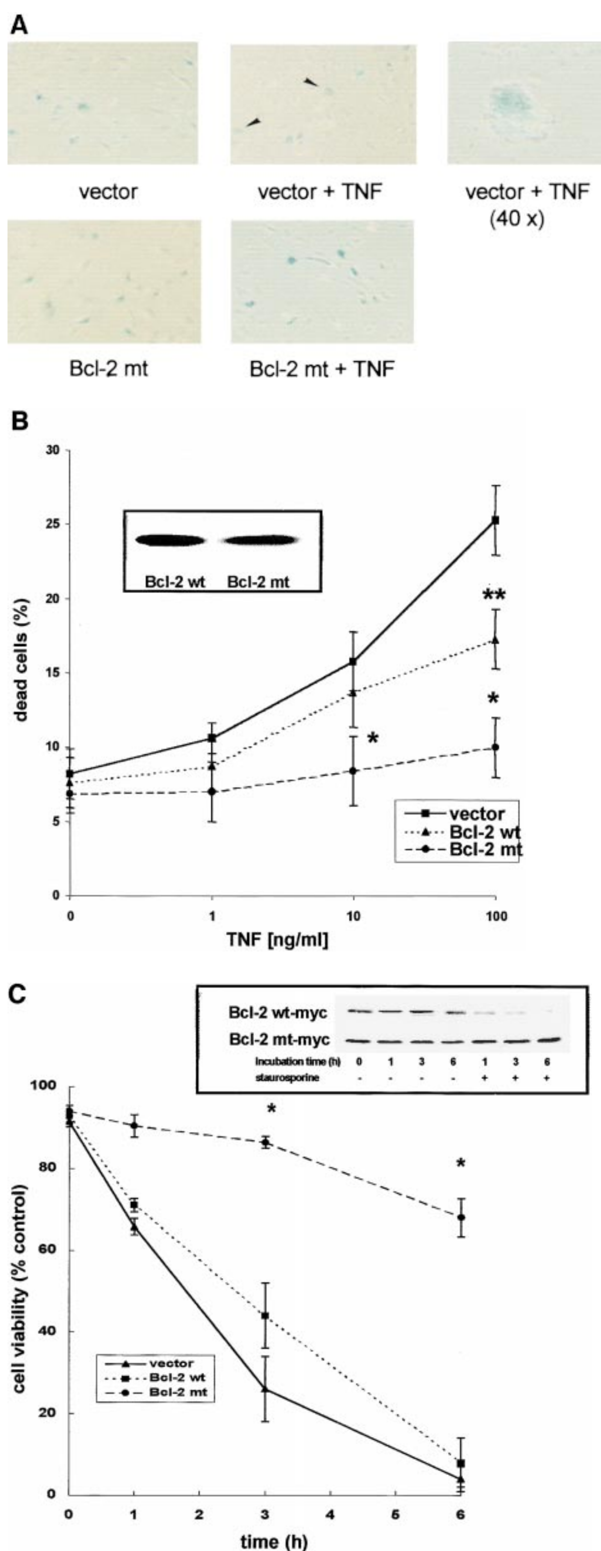

Figure 3. Influence of Bcl-2 degradation on apoptosis. (A and B) HUVECs were transiently cotransfected with pcDNA3.1-myc–wild-type Bcl-2 (Bcl-2 wt; 2 μg), pcDNA3.1-myc–Bcl-2 mutant lacking all four lysine residues (Bcl-2 mt; 2 μg), or control vector (pcDNA3.1 without insert; 2 μg) and pcDNA3.1-lacZ (1 μg). After incubation for 24 h to allow expression of the proteins, apoptosis was induced by incubation with TNF-α (100 ng/ml) for 18 h. Transfected cells were identified by β-galactosidase staining. Top panel (enlargement 1:10) shows a representative overview, with arrows indicating the dead cells. Dead cells were additionally analyzed at 1:40 magnification (top right panel) to confirm the morphological alterations typical for apoptotic cell death. (B) Dead versus viable cells were counted under phase–contrast by two independent investigators in a total number of 600 cells (data are mean ± SEM, $n = 4$–6; *$P < 0.05$ vs. Bcl-2 wt + TNF; **$P < 0.05$ vs. Bcl-2 mt + TNF). As shown in the insert, expression of the myc-tagged Bcl-2 wild-type and Bcl-2 mutant was controlled by Western blot with anti-myc antibodies. (C) Effect of Bcl-2 wild-type or Bcl-2 mutant lacking all four lysine residues on staurosporine-induced apoptosis. HUVECs were transiently transfected with the vectors described above, and apoptosis was stimulated with staurosporine (1 μM) for the indicated times. The basal expression and the degradation of the transfected Bcl-2 protein were determined by Western blot with anti-myc antibodies, as shown in the insert.

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Dephosphorylation of Bcl-2 Signals Ubiquitin-dependent Degradation. Targeting of proteins for ubiquitin-dependent degradation is often regulated by phosphorylation or dephosphorylation of the target protein (27, 30). Since previous studies suggested that reduction of the activity of MAP kinases ERK1/2 may be linked to dephosphorylation of Bcl-2 (37), we investigated whether the MAP kinase pathway is involved in the Bcl-2 degradation process. Prolonged incubation with TNF-α induced a drastic dephosphorylation and deactivation of ERK1/2 (Fig. 4 A; data not shown). Moreover, inhibition of ERK1/2 activity by the MAP kinase/ERK kinase (MEK) inhibitor PD98059 triggered the degradation of Bcl-2 (Fig. 4 B) and induced apoptotic cell death (data not shown), suggesting that inhibition of ERK1/2 may trigger the degradation of Bcl-2. Furthermore, activated ERK induced phosphorylation of Bcl-2 in vitro (Fig. 4 C). ERK-induced Bcl-2 phosphorylation was prevented by mutation of the three residues matching the consensus sequence for the putative MAP kinase sites (P-X-X-T/S-P) in Bcl-2 into alanine (Fig. 4 C), demonstrating the specificity of the reaction.

To test whether phosphorylation of Bcl-2 might prevent its degradation, we specifically mutated the three MAP kinase sites (P-X-X-T/S-P) into phospho-mimetic aspartic acid residues, which mimics continuous phosphorylation of the protein. Expression of the phospho-mimetic mutants completely prevented TNF-α–induced Bcl-2 degradation and apoptosis (Fig. 4 D). Thus, mimicking phosphorylation of putative MAP kinase sites within Bcl-2 is associated with complete inhibition of TNF-α–induced degradation. Finally, we tried to more precisely characterize the role of ERK to interfere with Bcl-2 degradation. Therefore, we assessed the effect of the ERK-specific phosphatase MKP-3. Since MKP-3 is exclusively located in the cytosolic compartment of cells, whereas MKP-1 and MKP-2 are localized in the nuclei (38, 39), MKP-3 may be one possible effector enzyme inducing the dephosphorylation of ERK. Overexpression of MKP-3 not only reduced ERK1/2 activity as described previously (data not shown; reference 38) but also induced degradation of wild-type Bcl-2 and subsequent apoptotic cell death (Fig. 4 E). In contrast, the phospho-mimetic Bcl-2 mutant or the Bcl-2 construct lacking all four lysine residues was resistant to MKP-3–triggered degradation (Fig. 4 E).

Taken together, ubiquitin-dependent proteolytic degradation decreases Bcl-2 protein levels and thereby renders

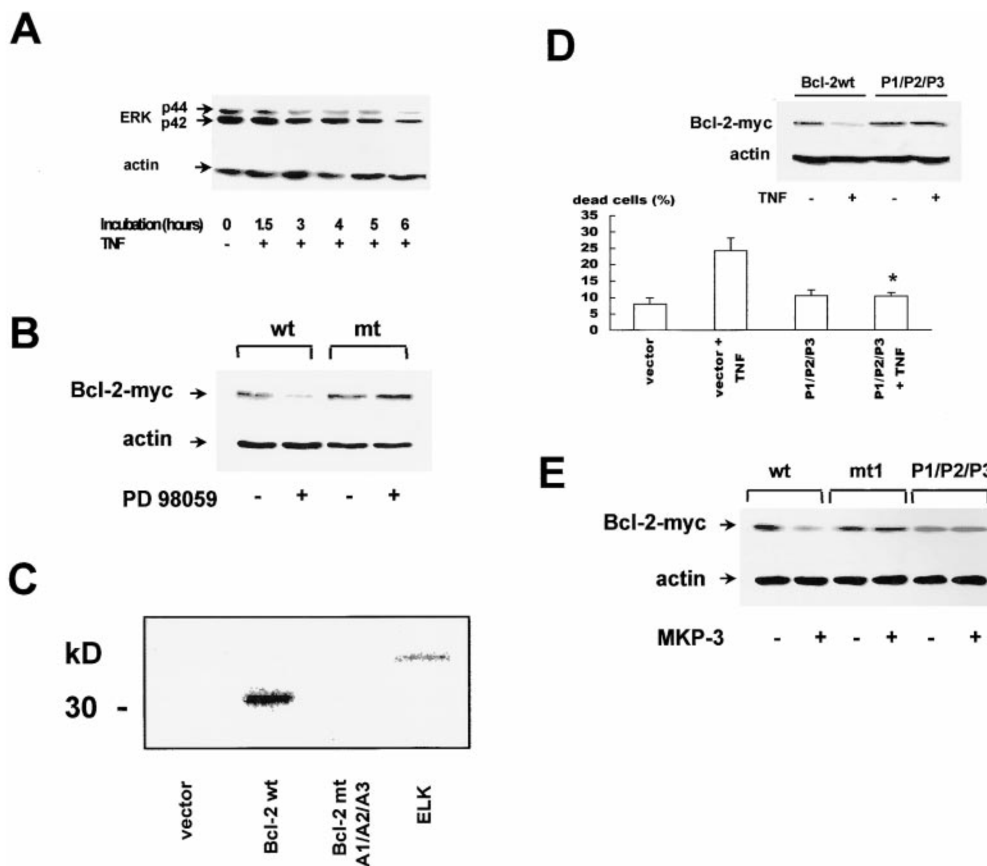

Figure 4. Involvement of MAP kinases in regulating Bcl-2 degradation. (A) Effect of TNF-α on ERK1/2 phosphorylation in HUVECs. ERK1/2 phosphorylation was determined by Western blot with a phospho-specific ERK1/2 antibody. The reduction of phosphorylation correlated with reduced enzyme activity (data not shown). Blots were reprobed with actin as a loading control. (B) Effect of PD98059 (10 μM; 12 h) on degradation of Bcl-2 wild-type (wt) and Bcl-2 mutant (mt) lacking all four lysine residues in HUVECs. (C) Phosphorylation of Bcl-2 by active ERK. Bcl-2 wild-type or Bcl-2 mutant lacking the putative MAP kinase acceptor amino acids A1/A2/A3 (T56A, T74A, S87A) were expressed in COS-7 cells, and myc-tagged Bcl-2 was immunoprecipitated. The immunoprecipitates or the control substrate Elk were incubated with [^{32}P]ATP and active MAP kinase for 30 min, then resolved on 12% SDS gels. An autoradiograph of the SDS gel is shown. (D) Effect of phospho-mimetic mutation of MAP kinase phosphorylation sites of Bcl-2 (P1, T56D; P2, T74D; P3, S87D) on TNF-α–stimulated Bcl-2 degradation and apoptosis. Plasmids (pcDNA3.1) encoding the myc-tagged mutants were transfected in HUVECs for 24 h to allow protein expression before stimulation with TNF-α for 12 h. Blots were reprobed with actin as a loading control. For detection of apoptosis, HUVECs were transiently cotransfected with 2 μg plasmids and 1 μg pcDNA3.1–β-galactosidase. Dead versus viable cells were counted under phase–contrast by two independent investigators in a total number of 600 cells (*$P < 0.05$ vs. vector + TNF and Bcl-2 wt + TNF; data are mean ± SEM, $n = 3$). (E) Effect of MKP-3 overexpression on Bcl-2 degradation in HUVECs. Plasmids (pcDNA3.1) encoding MKP-3 or control vector were transiently overexpressed in combination with the myc-tagged Bcl-2 wild-type or Bcl-2 mutants (mt: K17R, K22R, K218R, K239R, or P1/P2/P3: T56D, T74D, S87D). After 24 h, Bcl-2 wild-type or mutant protein levels were detected by Western blot with anti-myc antibodies. A reprobe with actin serves as loading control.

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cells susceptible for apoptotic stimuli. Moreover, our data may suggest that dephosphorylation of the putative MAP kinase sites targets Bcl-2 for ubiquitin-dependent degradation, whereas simulation of continuous phosphorylation of the putative MAP kinase phosphorylation sites of Bcl-2 not only abolishes its degradation, but—more important—confers resistance to stimulus-induced apoptosis. Bcl-2 is believed to control the activation of the caspase cascade by participation in a multiprotein "apoptosome" ensemble involving Apaf-1, cytochrome C, and caspase-9 (40). Thus, degradation of Bcl-2 may unleash the inhibitory function of Bcl-2 over the apoptosome. This is further evidenced by the finding that antisense-induced selective downregulation of Bcl-2 triggers the release of cytochrome C from the mitochondria (data not shown). Thus, ubiquitin-dependent degradation of Bcl-2 may represent an alternative pathway to amplify the caspase cascade. The activation of the ubiquitin-dependent proteasome complex leading to selective degradation of Bcl-2 not only appears to be a key signaling pathway used by TNF-α to amplify its potency to induce apoptosis, but may be involved in apoptosis signaling in all cells, where the mitochondrial amplification loop is important. Moreover, since the endothelium plays a pivotal role as a gatekeeper during inflammation, the selective downregulation of the antiapoptotic Bcl-2 by inflammatory cytokines such as TNF-α may significantly affect endothelial integrity and thus the progression of inflammatory diseases such as atherosclerosis. Inhibition of the signaling pathways involved in Bcl-2 degradation may not only provide insights into the pathophysiological role of Bcl-2 degradation, but may also have important novel therapeutic implications in disease states with deregulated apoptosis.

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