Defects in the Ubiquitin Pathway Induce Caspase-independent Apoptosis Blocked by Bcl-2*

(Received for publication, June 6, 1997, and in revised form, November 6, 1997)

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Aptosis requires the activation of caspases (formerly interleukin 1β-converting enzyme-like proteases), in particular those related to the caspase-3/7/8 subfamily. Recent data, however, revealed that, although caspase-specific inhibitors delay apoptosis, they are often incapable of preventing it. To obtain evidence for caspase-independent steps of apoptosis, we artificially created a high amount of short-lived or aberrant proteins by blocking the ubiquitin degradation pathway. A temperature-sensitive defect in the ubiquitin-activating enzyme E1 induced apoptosis independent of the activation of caspase-3 and -6 and the cleavage of their respective substrates poly(ADP-ribose) polymerase and lamin A. In addition, neither the caspase 3/7-specific inhibitor N-benzoylcarbonyl-Asp-Glu-Val-Asp-fluoromethylketone nor the general caspase inhibitor N-benzoylcarbonyl-Val-Ala-Asp-fluoromethylketone were capable of blocking this type of cell death. By contrast, Bcl-2 overexpression effectively protected cells from apoptosis induced by a defect in the E1 enzyme at the nonpermissive temperature. Bcl-2 acted downstream of the accumulation of short-lived or aberrant proteins because it did not prevent the overexpression of the short-lived proteins p53, p27kip1, and cyclins D1 and B1 under conditions of decreased ubiquitination. These results suggest the existence of short-lived proteins that may serve the role of caspase-independent effectors of apoptosis and attractive targets of the death-protective action of Bcl-2.

Programmed cell death (apoptosis) is an essential process to maintain homeostasis in multicellular organisms. It is triggered by a variety of physiological and nonphysiological agents that generate a common set of morphological alterations such as cell shrinkage, surface blebbing, chromatin condensation, DNA and nuclear fragmentations, and the formation of membrane-enclosed apoptotic bodies that are phagocytosed by neighboring cells (1).

The apoptotic process can be divided into three, functionally distinct phases: initiation, effector, and degradation (2). The initiation phase involves the activation of heterogeneous intracellular signaling pathways that are distinct ("private") for each death stimulus. These pathways converge on a common effector phase that, in turn, executes death by degrading various cellular components (3). The crucial molecular players in these phases are a series of cysteine proteases that are homologues of the interleukin 1β-converting enzyme (4). Unlike other cysteine proteases, these enzymes cleave their substrates following aspartate residues and have therefore been named caspases (5). All caspases exist as zymogens that require cleavage at internal aspartate residues to generate two-subunit active enzymes (6). Because of this property, they can activate each other and form an amplified protease cascade similar to that seen in clotting and complement activation (6). Most caspases are organized in "private" signaling pathways because their deletion or inhibition by specific inhibitors (Ac-YVAD-CHO, cytokine response modifier A (CrmA)) does not interfere with all sorts of apoptosis (7–9). However, at least three caspases, caspase-3, -6, and -7, seem to participate in the common death effector phase of apoptosis (5, 10–12). They are the closest homologues of the Caenorhabditis elegans ced-3 gene product shown to be essential for apoptosis during nematodal development (13). Moreover, specific peptide inhibitors of caspase-3 and -7 such as Z-DEVD-fmk1 interfere with most, if not all, forms of mammalian apoptosis. This suggests that the caspases of the death effector phase have been evolutionarily conserved, but that higher organisms have adopted variations of these enzymes to regulate "private" initiation phases as well. Numerous substrates of caspase-3, -7, and -6 have been identified in mammalian cells. Among them are poly(ADP-ribose) polymerase (PARP), a repair enzyme preferentially cleaved by caspase-3 and -7 (14), and lamin A, a component of the nuclear lamina, preferentially cleaved by caspase-6 (15, 16).

Genetic studies in C. elegans further uncovered a gene product called ced-9, which has an inhibitory effect on apoptosis during nematodal development (13). The mammalian homologue of ced-9 is Bcl-2, a proto-oncogene product that was originally identified at a t(14;18) chromosomal breakpoint common to human follicular lymphomas (17). Bcl-2 can functionally substitute for ced-9 and prevent nematodal (17) as well as mammalian apoptosis induced by various, often unrelated

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* This work was supported by Grants 31-36152.92 and 31-34600.92 from the Swiss National Science Foundation, Grant 421 from the Swiss Cancer League, and a grant from the Foundation for Aging Research (all to C. B.); by Grant AG04821 from the NIA, National Institutes of Health (to H. L. O.); and by Grant GM34009 from the National Institutes of Health (to A. L. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: Z-DEVD-fmk, N-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone; CHX, cycloheximide; DAPI, 4',6'-diamidino-2-phenylindole; MG132, carboxenoxyl-leucyl-leucyl-leucyl-leucyl-Val-Ala-Asp-fluoromethylketone; PAGE, polyacrylamide gel electrophoresis; Pipes, 1,4-piperazinediethanesulfonic acid; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase.
death stimuli (18). Both, genetic and biochemical studies revealed that ced-9 and Bcl-2 act upstream of ced-3 and caspase 3, respectively, to block caspase activation and apoptosis (13, 19–21). However, neither ced-9 nor Bcl-2 directly interact with their respective caspases. The link has been provided by ced-4, another gene product essential for apoptosis in C. elegans (13). ced-4 directly binds to and activates ced-3/caspases and triggers nematodal and mammalian apoptosis in a ced-3-dependent manner (13, 22, 23). Simultaneously, ced-4 interacts with ced-9/Bcl-2 and allows the latter to block the activation of ced-3/caspases (22–24). Thus, ced-4 has a double impact on the effector phase of apoptosis; it can act as a caspase activator and as an adapter for Bcl-2 to inhibit caspases. A further characterization of the function of ced-4 has recently become available through the cloning of a mammalian homologue, called Apaf-1 (25).

On the search for mammalian activators of caspases such as ced-4 homologues, we made the following assumption. If these activators were constitutively expressed, as it had been proposed (26), their death-promoting activities should be dormant in surviving cells. Any death stimulus would have to reactivate them by posttranslational modifications or the binding or release of regulatory proteins. An attractive, but often overlooked alternative is changes in the protein half-lives of the caspase activators. Since the ubiquitin system is a prominent regulator of protein turnover, we tested whether defects in this system would lead to caspase activation or apoptosis.

Ubiquitin-dependent degradation is necessary to eliminate damaged, misfolded proteins as well as proteins whose impact on biologic processes has to be of short duration (short-lived proteins) (27). In this pathway, ubiquitin (Ub) is first activated in an ATP-dependent manner by a thiolester linkage to the Ub-activating enzyme, E1. Activated Ub is then transferred to one of several Ub-conjugating enzymes, E2, to form another thiolester intermediate. From E2, Ub is ligated to lysine residues of target substrates sometimes with the help of specific E3 Ub protein ligases. Further ubiquitination of the mono-Ub protein substrates leads to the formation of multi-Ub chains that are recognized by the 26 S proteasome for degradation. An essential protease of the 26 S proteasome has recently been found to have a threonine residue in its active center. This site has subsequently been identified as the target for the specific proteasomal inhibitor lactacystin (28). While several E2 enzymes exist, only one E1 enzyme has been found thus far in mammalian cells (27). Thus, inactivation of the E1 enzyme abrogates ubiquitination and leads to an accumulation of aberrant or short-lived protein (29). This is accentuated by a further blockage of the proteasome by lactacystin.

We show here by using a cellular system of diminished E1 activity and simultaneous proteasomal inhibition that the turnover of short-lived proteins plays a crucial role in the apoptotic response. When present in high amounts, these proteins seem to activate a caspase-independent type of apoptosis that is still effectively impeded by Bcl-2 overexpression.

MATERIALS AND METHODS

Reagents—Hygromycin B, cycloheximide (CHX), Hoechst 33342, and Z-DEVD-fmk were purchased from Juro Supply/Calbiochem and DNase-free RNase from Boehringer Mannheim. Polyvinylidene difluoride (PVDF) membranes (Immobilon-P) were from Millipore, and the enhanced chemiluminescence (ECL) detection system was from Amer sham. Z-VAD-fmk was from Enzyme System Prod., and MG132 was provided by Proscript Inc. The LIVE/DEAD fluorometric assay was purchased from Molecular Probes.

Cells—The temperature-sensitive ts20 Balb/C 3T3 clone A3 fibroblast cell line and its E1-corrected H38-5 derivative were cultured as described previously (29). Vector control (pMV12) and Bcl-2-overexpressing (Bcl-2) ts20 cells were generated by retroviral transduction of the pMV12hygro plasmid lacking or containing the murine Bcl-2 cDNA.
A Defect in the E1 Enzyme Leads to Growth Arrest—ts20 is a derivative of Balb/c 3T3 fibroblasts that harbors a temperature-sensitive defect in the E1 ubiquitin-activating enzyme (29). At the nonpermissive temperature (39 °C), E1 enzyme activity is largely decreased, protein ubiquitination wanes and the cells are arrested at G1 or G2/M phases (see also Fig. 6). To rescue ts20 cells from the defects of ubiquitination and growth, H38-5 cells were generated by transfecting a wild-type allele of E1 into ts20 cells at 39 °C (Fig. 1A). At 34 °C, both H38-5 and ts20 cells displayed exponential growth characteristics over a time period of 4–7 days (Fig. 1A). The rate of proliferation was slower for H38-5, presumably due to a clonal effect (Fig. 1A). Upon shifting the temperature to 39 °C, H38-5 cells continued to proliferate while ts20 cells declined in number after an initial increase during the first 24 h (Fig. 1B).

A Defect in the E1 Enzyme Induces Apoptosis—The loss of ts20 cells at 39 °C was indicative of cell death in addition to growth arrest. Indeed, after 24 h at 39 °C, the ts20 cells (data not shown) and its vector control analog ts20pMV12 (Fig. 2A) exhibited morphological changes typical of apoptosis such as cell shrinkage and plasma membrane blebbing. In addition, the cells detached from the plate, stained with trypan blue, and displayed chromatin condensation, the fragmentation of nuclei (Fig. 2C), and the cleavage of genomic DNA into nucleosome-sized fragments (Fig. 2B). None of these changes were observed with ts20 and ts20pMV12 cells at 34 °C or H38-5 at both 34 °C and 39 °C at any time (Fig. 2 and data not shown). This indicates that a defect in the E1 enzyme provokes apoptosis.

Overexpression of Bcl-2 Delays Apoptosis Due to a Defective E1 Enzyme—To examine whether Bcl-2 interfered with apoptosis induced by a defective E1 enzyme, we infected ts20 cells with a retrovirus containing the murine Bcl-2 cDNA (ts20Bcl-2). In parallel, vector control cell lines were generated (ts20pMV12). Immunoblot analysis revealed various degrees of overexpression of the 26-kDa Bcl-2 in stable ts20Bcl-2 cell lines at both 34 °C and 39 °C (Fig. 1C). In addition, as shown by immunocytochemistry, the overexpressed Bcl-2 was correctly localized to mitochondrial, endoplasmic reticulum, and nuclear membranes (data not shown).

First, we measured the proliferation of ts20pMV12 and ts20Bcl-2 cells by counting viable (trypan-excluding) cells. At 34 °C, ts20Bcl-2#7 and ts20Bcl-2#13 cells proliferated slightly slower than the ts20pMV12 vector control cell line (Fig. 1A). As the parental cells, the vector control cells decreased in number after 24 h at 39 °C (Fig. 1A). On the other hand, both the ts20Bcl-2#7 and ts20Bcl-2#13 cell lines continued to proliferate at 39 °C although never as much as the E1-expressing H38-5 cells. Whereas the low Bcl-2-expressing cell line ts20Bcl-2#13 showed some cell loss after 48 h at 39 °C, this was not the case for the high Bcl-2-expressing ts20Bcl-2#7. Even after 7 days, the latter cells were still alive, although they did not further proliferate (Fig. 1B). At 48 h, no morphologic changes, chromatin condensation or nuclear or DNA fragmentation were seen in ts20Bcl-2#7 cells, whereas all these properties were evident in the ts20pMV12 vector control (Fig. 2). These data indicate that Bcl-2 partially rescues ts20 cells from growth inhibition and apoptosis at the nonpermissive temperature.

Apoptosis Induced by a Defective E1 Enzyme Is Independent of Caspase Activation—Next, we investigated the molecular mechanisms underlying apoptosis due to a defective E1 enzyme. For that purpose, we monitored the activation of caspase-3 and -6 and the cleavages of their respective substrates PARP and lamin A in ts20pMV12 cells at 39 °C for 72 h. Western blot analyses revealed that neither the 32-kDa proform of caspase-3 nor the 34-kDa proform of caspase-6 was converted into, respectively, active 17-kDa or 21-kDa species at 39 °C. Moreover, neither PARP nor lamin A were cleaved (Fig. 3A and B, left panels). In addition, no cleavages of PARP (Fig. 3A) or lamin A (Fig. 3B) were detected. Since PARP is also a known substrate for caspase-7 (11), none of the effector caspases appeared to be activated during apoptosis due to a defect in the E1 enzyme. ts20pMV12 cells, however, displayed efficient maturation/activation of caspase-3 and caspase-6, as well as cleavages of PARP and lamin A upon apoptosis induced by staurosporine at 34 °C (data not shown) and 39 °C (Fig. 3A, A and B, right panels), indicating that the cells were not deficient in activating the caspase execution program when exposed to appropriate apoptotic stimuli.

To further explore a possible involvement of caspase-3, -7, or...
-6 activation in apoptosis due to a defect in the E1 enzyme, we treated the cells with the cell-permeable tetrapeptide caspase inhibitors Z-DEVD-fmk and Z-VAD-fmk. These inhibitors have been shown previously to block various forms of apoptosis by binding as pseudo-substrates to the catalytic site of caspases (3, 10). Whereas Z-DEVD-fmk preferentially inhibits caspase-3 and -7 due to the aspartic acid (D) at the P-4 position, Z-VAD-fmk is more promiscuous and blocks all so far identified caspases, including caspase-6, at concentrations >50 μM (3). ts20pMV12 cells at 39 °C were pretreated and retreated every 24 h with 100 μM Z-VAD-fmk or Z-DEVD-fmk (Figs. 3 and 4). Apoptotic morphologies (Fig. 4A), chromatin condensation (Fig. 4B), and the number of trypan blue-stained, detached cells (Fig. 1B) were similar between untreated and inhibitor-treated cells at any time during apoptosis at 39 °C. This was not due to a lack of action of the inhibitors in ts20 cells as both Z-VAD-fmk and Z-DEVD-fmk completely prevented caspase-3 and -6 activations and PARP and lamin A cleavages in response to staurosporine at 39 °C (Fig. 3, A and B). These data indicate that apoptosis induced by a defective E1 enzyme does not involve the activation of caspases. By consequence, Bcl-2 must also use targets other than caspases to exert its death-protective effect.

Bcl-2 Neither Acts as E1 enzyme Nor Diminishes the Accumulation of Short-lived/Aberrant Protein at 39 °C—To unravel how Bcl-2 protects ts20 cells from apoptosis induced by a defective E1 enzyme, we investigated the effect of Bcl-2 on the...
ubiquitin/proteasome degradation system. As shown by anti-ubiquitin Western blotting, a defect in E1 at 39 °C caused a marked diminution of overall protein ubiquitination, including that of histones and high molecular weight proteins (Fig. 5, A and B). Although this reduction was not complete due to residual E1 activity, it was sufficient to provoke the massive accumulation of short-lived proteins such as p53, the cyclin-dependent protein kinase (cdk) inhibitor p27kip1, and the cyclins D1 and B1 (Fig. 5, C, D, and F), all known targets of the ubiquitin/proteasome system (29, 36–39). While p53, p27kip1, and cyclin B1 control entry into the S and M phases, respectively, cyclin D1 primarily stimulates cell cycle progression during the G1 phase (36, 37, 40–42). Accumulation of these proteins perturbs cell cycle homeostasis and leads to cell cycle arrests or apoptosis (45). Whether this is due to a degradation by the ubiquitin/proteasome system has not yet been established. We performed an anti-Bax Western blot of H38-5, ts20pMV12, ts20Bcl-2#7, and ts20Bcl-2#13 extracts obtained from cells either grown at 34 °C or 39 °C. Fig. 5E shows that both H38-5 and ts20 cells express endogenous 21-kDa Bax at 34 °C. However, this level did not increase in either cell derivative at the nonpermissive temperature. This indicates that Bax is not degraded by the ubiquitin/proteasome system (Fig. 5E) and that apoptosis of ts20 cells at 39 °C is not due to an accumulation of the Bax protein.

Bcl-2 Bypasses G1/S and G2/M Cell Cycle Checkpoints—Our finding that ts20Bcl-2#7 cells still proliferated (Fig. 1B) in the presence of high levels of p53, p27kip1, and cyclin B1 (Fig. 5, C, D, and F) suggested that Bcl-2 may have been able to disrupt G1/S or G2/M cell cycle checkpoints. To provide further evidence for this notion, we determined the cell cycle profile of ts20pMV12 and ts20Bcl-2#7 cells at both 34 °C and 39 °C. We used a flow cytometry method on DAPI-stained DNA (31) that allowed the simultaneous analysis of apoptotic cells with fragmented DNA (sub-G1 peak fraction) and nonapoptotic cells in various cell cycle states (DNA ploidy). At 34 °C, both ts20pMV12 and ts20Bcl-2#7 exhibited cell cycle profiles typical

Apoptosis Induced by Decreased Ubiquitination Is Not Due to an Accumulation of the Pro-apoptotic Bax Protein—Having established that Bcl-2 does not prevent the accumulation of short-lived/aberrant proteins at the nonpermissive temperature, we wanted to know whether expression of its pro-apoptotic partner Bax was enhanced as well. There is emerging evidence that increased Bax expression sensitizes cells for apoptosis (44). In addition, as compared with Bel-2, which is an extremely stable protein, Bax has been shown to exhibit a high turnover (45). Whether this is due to a degradation by the ubiquitin/proteasome system has not yet been established. We performed an anti-Bax Western blot of H38-5, ts20pMV12, ts20Bcl-2#7, and ts20Bcl-2#13 extracts obtained from cells either grown at 34 °C or 39 °C. Fig. 5E shows that both H38-5 and ts20 cells express endogenous 21-kDa Bax at 34 °C. However, this level did not increase in either cell derivative at the nonpermissive temperature. This indicates that Bax is not degraded by the ubiquitin/proteasome system (Fig. 5E) and that apoptosis of ts20 cells at 39 °C is not due to an accumulation of the Bax protein.

Caspase-independent Apoptosis Blocked by Bcl-2—Having established that Bcl-2 does not prevent the accumulation of short-lived/aberrant proteins at the nonpermissive temperature, we wanted to know whether expression of its pro-apoptotic partner Bax was enhanced as well. There is emerging evidence that increased Bax expression sensitizes cells for apoptosis (44). In addition, as compared with Bel-2, which is an extremely stable protein, Bax has been shown to exhibit a high turnover (45). Whether this is due to a degradation by the ubiquitin/proteasome system has not yet been established. We performed an anti-Bax Western blot of H38-5, ts20pMV12, ts20Bcl-2#7, and ts20Bcl-2#13 extracts obtained from cells either grown at 34 °C or 39 °C. Fig. 5E shows that both H38-5 and ts20 cells express endogenous 21-kDa Bax at 34 °C. However, this level did not increase in either cell derivative at the nonpermissive temperature. This indicates that Bax is not degraded by the ubiquitin/proteasome system (Fig. 5E) and that apoptosis of ts20 cells at 39 °C is not due to an accumulation of the Bax protein.

Bcl-2 Bypasses G1/S and G2/M Cell Cycle Checkpoints—Our finding that ts20Bcl-2#7 cells still proliferated (Fig. 1B) in the presence of high levels of p53, p27kip1, and cyclin B1 (Fig. 5, C, D, and F) suggested that Bcl-2 may have been able to disrupt G1/S or G2/M cell cycle checkpoints. To provide further evidence for this notion, we determined the cell cycle profile of ts20pMV12 and ts20Bcl-2#7 cells at both 34 °C and 39 °C. We used a flow cytometry method on DAPI-stained DNA (31) that allowed the simultaneous analysis of apoptotic cells with fragmented DNA (sub-G1 peak fraction) and nonapoptotic cells in various cell cycle states (DNA ploidy). At 34 °C, both ts20pMV12 and ts20Bcl-2#7 exhibited cell cycle profiles typical...
of adherent, asynchronously growing fibroblasts, i.e. 60% in G1, 23% in S, and 17% in G2/M (Fig. 6). As shown previously (29), ts20pMV12 cells were selectively lost from the S phase and majorly accumulated in the G1 and G2/M phase at 36 h after shift to 39 °C (Fig. 6). In addition, up to 50% of the cells displayed a DNA stainability lower than that of G1 cells, indicating that they contained low molecular weight DNA characteristic of apoptotic cells. Interestingly, none of these changes were seen with ts20Bcl-2#7 cells (Fig. 6). A significant proportion of these cells (20–25%) were still found in the S phase at 39 °C, indicating that they were cycling and thus bypassing the cell cycle checkpoints usually imposed by accumulating levels of p53, p27kip1, or cyclin B1.

Cycloheximide Prevents Accumulation of Short-lived proteins

![Graph](image)

Fig. 5. Decrease in ubiquitination, accumulation of short-lived proteins and unaltered Bax at 39 °C. The various ts20 cell derivatives were grown on 10-cm plates at 34 °C for 24 h before being shifted to 39 °C or reincubated at 34 °C for 48 h. Following total protein extraction, equal amounts of protein were subjected to 12% SDS-PAGE, blotted to PVDF, and probed with anti-ubiquitin (A and B), anti-p53 (C), anti-p27kip1 (D), anti-Bax (E), or anti-cyclin D1 and B1 (F) antibodies. Proteins were quantified by imaging densitometry of the ECL autoradiography (A–D). The displayed values are the means of three independent experiments ± S.D.

Caspase-independent Apoptosis Blocked by Bcl-2
at 39 °C and Blocks Apoptosis Induced by Decreased Ubiquitination—The data so far are consistent with the idea that one or several short-lived proteins that accumulate in response to the inactivation of the E1 enzyme cause apoptosis. To support this hypothesis, we pretreated ts20pMV12 cells with cycloheximide for 2 h before exposing them to 39 °C. Because short-lived proteins are rapidly lost in cycloheximide-treated cells, they would not be available to trigger apoptosis at 39 °C. We found that this is indeed the case. ts20pMV12 cells pretreated with 5 μg/ml cycloheximide and then grown at 39 °C in the presence of lower concentrations of cycloheximide (1 μg/ml), did not accumulate short-lived proteins such as p53 (Fig. 7B) and did not die by apoptosis, as judged by morphological changes (Fig. 2A), chromatin condensation (Fig. 2C), and DNA fragmentation (Fig. 2D). Cycloheximide even had an effect when given after apoptosis induction at 39 °C. In this case, depending on the time of cycloheximide addition, p53 (Fig. 7D) rose to detectable levels, but apoptosis was still delayed (data not shown). Because less protein was synthesized in cycloheximide-treated cells, it was less ubiquitinated than in untreated cells at both 34 °C and 39 °C (Fig. 7, A and C). These results indicate that high levels of short-lived proteins are required to promote apoptosis. If their expression is diminished due to cycloheximide treatment, their apoptotic activity is reduced markedly.

Bcl-2 Protects against Apoptosis Induced by the Simultaneous Inhibition of Ubiquitination and Proteosomal Degradation—How does Bcl-2 neutralize the action of apoptotic short-lived proteins? It may direct these proteins to the proteasomal or lysosomal degradation system without prior ubiquitination, although such a mechanism does not seem to account for p53, p27kip1, and the cyclins D1 and B1 (see Fig. 5, C, D, and F). We tested this hypothesis by blocking proteasomal or lysosomal activity using respective inhibitors. Treatment of ts20pMV12 cells with 5 μM amounts of the proteasome inhibitor lactacystin (28) markedly accelerated apoptosis at 39 °C (Fig. 8A). This can be explained by a further accumulation of short-lived proteins, which, in the absence of lactacystin, were still partially ubiquitinated and degraded due to an inefficient inactivation of the E1 enzyme at 39 °C. Importantly, ts20Bcl-2#7 and, to a lesser extent, ts20Bcl-2#13 cells were still protected from apoptosis induced by a lactacystin treatment at 39 °C (Fig. 8A). This cell loss was by apoptosis as the genomic DNA of MG132-treated ts20pMV12 cells was fragmented (data not shown). These results indicate that Bcl-2 does not bypass ubiquitination to directly stimulate the degradation of short-lived, apoptosis-promoting proteins by the proteasome or lysosomal systems.

Fig. 6. Bcl-2 bypasses G1/S and G2/M cell cycle arrests at 39 °C. ts20pMV12 and ts20Bcl-2#7 cells were grown on 10-cm plates at 34 °C for 24 h before being shifted to 39 °C for 36 h. The cells were then fixed in ethanol, rinsed with phosphate-citrate buffer stained with the DNA-specific fluorochrome DAPI, and analyzed by flow cytometry as described under “Materials and Methods.” Note that ts20pMV12 at 39 °C display a selective loss of S phase cells, an accumulation of cells in G1 and G2/M phases, and the appearance of cells with a fractional DNA content (sub-G1 peak). A small amount of sub-G1 cells is also detected with ts20Bcl-2#7 cells at 39 °C. However, no decrease of S phase cells is seen.
tems. It may instead inhibit the apoptotic activity of these proteins by direct protein-protein interactions.

**DISCUSSION**

The present study describes a novel, caspase-independent form of apoptosis that can be effectively delayed by Bcl-2 overexpression. The molecular players in this process appear to be short-lived or aberrant proteins that are usually degraded by the ubiquitin/proteasome system. Due to diminished ubiquitination, these proteins accumulate to levels that are probably sufficient to trigger apoptosis without the participation of caspases. Because Bcl-2 overexpression still allows these proteins to accumulate but not to trigger apoptosis, they represent attractive targets for the death-protective action of Bcl-2.

Ubiquitin-dependent degradation mainly serves to rid the cell of abnormal and misfolded proteins and to limit the time and amounts of availability of critical regulatory proteins (short-lived proteins) (27). This is essential for numerous cellular processes such as cell cycle control (38), gene transcription (47), chromatin maintenance (48), cytoskeletal (49) and surface receptor functions (50), intracellular trafficking (51), antigen presentation (52), and stress responses (53). It is therefore conceivable that defects in the ubiquitination pathway perturb cell homeostasis and, if becoming extensive, can promote cell death. The present data support this notion and

**FIG. 7.** Cycloheximide inhibits apoptosis, blocks p53 accumulation and decreases ubiquitination. The various ts20 cell derivatives were grown on 10-cm plates at 34 °C for 22 h, treated with 5 μg/ml cycloheximide for an additional 2 h at 34 °C, and then cultured at 39 °C or 34 °C in the presence of 1 μg/ml cycloheximide for 48 h (A and B). Alternatively, the cells were incubated at 34 °C for 24 h before being shifted to 39 °C or reincubated at 34 °C for 36 h, then treated with 5 μg/ml cycloheximide for 2 h and incubated at the respective temperature in the presence of 1 μg/ml cycloheximide for an additional 10 h (C and D). Following total protein extraction, equal amounts of protein were subjected to 15% SDS-PAGE, blotted to PVDF, and probed with anti-ubiquitin (A and C) or anti-p53 (B and D) antibodies. Proteins were quantified by imaging densitometry of the ECL autoradiography. The displayed values are the means of three independent experiments ± S.D.
confirm previous reports showing that proteasomal inhibition induced apoptosis (54, 55) and an up-regulation of ubiquitination prevented cell damage generated by heat shock or hypoxia (ischemia) (53, 56).

Nevertheless, pro-apoptotic activities of the ubiquitin system have also been reported. Muscle cell death in the hawkmoth Manduca sexta involved the transient up-regulation of ubiquitin mRNA (57, 58). This, however, occurred at a late stage of the process and probably served to eliminate cytoplasm before phagocytosis rather than to execute cell death. On the other hand, certain forms of apoptosis in neurons and thymocytes were shown to depend on a functional proteasome (59, 60), presumably because a recently identified proteasomal caspase was required to activate death effectors in an early phase of apoptosis (61). However, neurons and thymocytes do not differ from other cells in being susceptible to apoptosis when persistently exposed to proteasome inhibitors (59, 60).

Short-lived Proteins as Death Effectors or Inhibitors of Survival Factors—What might be the effector molecules that trigger apoptosis in response to decreased ubiquitination? Whereas aberrant proteins may interfere with vital cellular functions in a rather nonspecific way, accumulated short-lived proteins may have selective targets. Two mechanisms of action can be envisaged. First, short-lived proteins may be death effectors at high levels. They would be present in minute, nontoxic amounts in normal cells but accumulate to levels that activate some unknown ‘death substrates’ in cells with a defective E1 enzyme. If apoptosis is induced by other stimuli such as staurosporine, tumor necrosis factor α, or Fas/APO, the short-lived proteins may accumulate due to a posttranslational modification that prevents their ubiquitin-dependent degradation. Various transcription factors such as p53, Myc, Jun, E2F, and cell cycle components such as the cyclins and cdk25 are rapidly degraded by the ubiquitin degradation pathway following phosphorylation, dephosphorylation, or other modifications (41, 62–66). Interestingly, most of these proteins promote apoptosis when accumulating or forced to be overexpressed (43, 67–71). It is, however, unknown how their usual function as cell cycle or transcriptional regulators is converted into an apoptotic activity. According to the data here, this may be done by changing their half-lives. A similar mechanism has already been suggested for the conversion of these proteins into oncogene products (65) and indicates that the ubiquitin system may be an attractive, common regulator of tumorigenesis and apoptosis.

As an alternative to a direct death effector function, the short-lived proteins may inhibit crucial survival factors. A good example is the case of the transcription factor NFκB. This protein has recently been shown to protect cells from tumor necrosis factor α-induced apoptosis (72, 73), presumably via activating the transcription of genes whose products oppose apoptosis. NFκB is activated by the ubiquitin-dependent degradation of its inhibitory partner IκB (51). Thus, upon decreased ubiquitination, IκB or an analogous protein could accumulate and prevent NFκB or an analogous protein from acting as survival factor.

Short-lived Proteins as Targets of Bcl-2—In this study, Bcl-2 acts after the accumulation of aberrant or short-lived proteins to produce two cellular effects: protection against apoptosis and disruption of G1/S and G2/M cell cycle checkpoints, usually imposed by persistently high levels of p53, p27kip1, or cyclin B1, respectively. We do not yet know whether these two effects are causally linked or nor Bcl-2 achieves to regulate them. Three mechanisms can be envisaged.

(i) Bcl-2 directs accumulating proteins to a ubiquitin-independent proteolytic degradation system. Such ‘antizymes’ have been identified for p53 (E6) (47), cyclin A/B2 (p534–42c) (74), and ODC (ODC antizyme) (75) and serve to accelerate the degradation of the respective short-lived protein. The death substrates that may be “antizymed” by Bcl-2 are, however, distinct from p53, p27kip1, or the cyclins D1 and B1 because the latter accumulate to similar levels in vector control and Bcl-2-overexpressing cells. Moreover, the degradation machinery of these substrates are neither the proteasome nor lysosomes. Proteasomal inhibitors as well as lysosomotropic agents enhance the accumulation of short-lived proteins and provoke apoptosis much faster than decreased ubiquitination alone, but Bcl-2 is still effectively death-protective. These data do not, however, exclude the possibility that Bcl-2 exploits another proteolytic system to degrade specific short-lived death effectors. Moreover, Bcl-2 may sequester short-lived proteins into lysosomes where they are not degraded but unable to trigger apoptosis.

(ii) Alternatively, Bcl-2 converts aberrant or short-lived proteins into an apoptosis-incompetent conformation. This could be best explained by a chaperoning function of Bcl-2. Chaperones like the well known heat shock proteins are ATPases that hold aberrant, denatured proteins in a nontoxic conformation. Bcl-2 cooperates with heat shock proteins to protect cells from lethal doses of heat shock (76), and certain functions of Bcl-2 such as its antioxidative activity can also be performed by heat shock proteins (77). However, Bcl-2 lacks an ATP binding site and may require other chaperones to effectively neutralize...
aberrant proteins (co-chaperone model). Such an activity of Bcl-2 would explain why this protein is “sticky” and interacts with numerous substrates such as R-Ras, Raf, the prion protein, Nips, and others (78), which cannot be easily defined as physiological partners of Bcl-2 (79). Apart from simply neutralizing aberrant protein, Bcl-2 may interact with specific short-lived death effector proteins and inhibit their apoptotic action. Two attractive targets are p53 and Bax. It has been reported that overexpressed p53 can induce apoptosis (70). Thus, the death described herein may be p53-dependent. Preliminary data, however, indicate that conditional overexpression of p53 in ts20 cells does not lead to apoptosis but to growth arrest (data not shown). In addition, the expression of Bax, a p53-inducible gene (80), does not increase upon p53 accumulation in ts20 cells at 39 °C. Thus, neither Bax nor p53 seem to be the targets of Bcl-2 and mediators of the apoptotic response induced by decreased ubiquitination.

(iii) Finally, Bcl-2 could replace a survival factor whose activity is blocked by decreased ubiquitination. As discussed above, the activity of the survival factor c-Rel/NFκB is controlled by the ubiquitination of its inhibitor IκB. Interestingly, a temperature-sensitive c-Rel induces apoptosis at the nonpermissive temperature that can be blocked by Bcl-2 overexpression (9). This indicates that Bcl-2 may compensate for the loss of anti-apoptotic gene products that are usually induced by c-Rel/NFκB.

Cycloheximide Effects Support the Involvement of Short-lived Proteins in Apoptosis—It has become widely accepted that the apoptotic effector machinery is constitutively expressed in mammalian cells (26). If all death effectors were stable proteins, a blockage of their synthesis by cycloheximide would not immediately affect their apoptotic potential. However, if some of them were short-lived proteins, they would be rapidly eliminated from the cell. This is what seems to happen with apoptosis induced by decreased ubiquitination or the additional treatment with proteasome inhibitors. A short-term cycloheximide treatment before apoptosis induction entirely abrogates the apoptotic response. Even when given shortly after apoptosis induction, cycloheximide still had a slight protective effect, presumably because aberrant or accumulated short-lived proteins failed to reach the apoptotic threshold level. These results are in agreement with previous data in neurons, where cycloheximide saved the cells from apoptosis due to nerve growth factor withdrawal up to a point of no return (approximately 18 h) (81). Based on the latter finding, it was proposed that the apoptosis-promoting factors were de novo synthesized death effector proteins. Here, we suggest an alternative view to this paradigm: the accumulation of pre-existing death effector proteins.

Caspase-independent Apoptosis Blockable by Bcl-2: Existence of Novel Death Effectors?—To our surprise, apoptosis induced by decreased ubiquitination was caspase-independent. This was because (i) the death effector proteases caspase-3 and -6 were not activated; (ii) PARP, a specific substrate of both effector caspases-3 and -7 and lamin A, a specific substrate of caspase-6, were not cleaved; and (iii) both Z-DEVD-fmk, a caspase-3/7-specific inhibitor, and Z-VAD-fmk, a general caspase inhibitor, were incapable of blocking this type of apoptosis. It is difficult to imagine that caspases exist that would not be blocked by any of these inhibitors. However, we still cannot exclude such a possibility. In this respect, it would have been informative to test another, well-known general caspase inhibitor, the baculovirus p35 protein (82), for its effect on this type of apoptosis; unfortunately, we did not succeed in achieving high level expression of this protein in ts20 cells. Irrespective of the possible involvement of unknown caspases, the apoptosis described herein was effectively blocked by Bcl-2. This shows for the first time that Bcl-2 targets molecules in addition to the presently known caspases to achieve death protection.

Recent reports have provided evidence for caspase-independent steps of apoptosis. (i) The overexpression of Bax activated caspase-3 but the resulting apoptosis was not blocked by Z-VAD-fmk (83). (ii) Degranulation-initiated target cell lysis by cytotoxic T cells could not be blocked by caspase inhibitors, yet DNA fragmentation could (84, 85). (iii) Nuclear fragmentation induced by DNA damage or by the overexpression of myc or Bak were blocked by Z-VAD-fmk, but other apoptotic features such as membrane blebbing persisted for days and the cells could not be rescued from the death fate (86). (iv) Bak and the C. elegans death gene product ced-4 induced cell death in yeast with morphological changes similar to those with apoptosis in mammalian cells, but no caspases have yet been described in yeast (87, 88). These data indicate that caspases do not control all aspects of apoptosis.

What might be the mechanisms of caspase-independent apoptosis? Based on the finding that Z-VAD-fmk did not prevent the rapid fall in the mitochondrial membrane potential in response to Bax overexpression (83), it was proposed that caspase-unrelated death effectors may perturb mitochondrial functions leading to an opening of the permeability transition (PT) pore or the release of pro-apoptotic factors such as cytochrome c and an apoptogenic protease (2). Indeed, mitochondrial perturbations are associated with many forms of apoptosis (2), and Bcl-2 can block the fall in the mitochondrial membrane potential as well as the appearance of cytochrome c and the apoptogenic protease in the cytoplasm (2, 89, 90). However, the treatment of ts20 cells with the PT pore blockers bongkrekic acid and cyclosporine A failed to delay apoptosis induced by decreased ubiquitination (data not shown), thus excluding the involvement of the PT pore in the caspase-independent steps of this type of apoptosis.

Another attractive, caspase-independent mediator of apoptosis is the nematodal ced-4 protein. Based on genetic analysis in C. elegans, the ced-4 protein participates in caspase (ced-3)-dependent and -independent forms of apoptosis that can both be blocked by ced-9/Bcl-2 (13). In addition, ced-4-induced (and, so far, ced-3/caspase-independent) chromatin condensation and killing in yeast is inhibited by direct physical association with ced-9 (88). The recent isolation of a mammalian homologue of ced-4, called Apaf-1 (25), will make it possible to test whether caspase-3 and -6 are necessary in apoptosis. (i) The overexpression of Bax activated death effector proteins. Here, we suggest an alternative view to this paradigm: the accumulation of pre-existing death effector proteins.

Acknowledgments—We are grateful to D. W. Nicholson for the anti-caspase 3 antibody p17, to G. G. Poirier for the anti-PARP, to R. I. Igo for the anti-p53 antibody Ab240, to Le Sun for the anti-Bax antibody, and to Proscript Inc. for MG132. We thank A. Conzelmann, S. Rusconi, J.-L. Dreyer, and C. Waeber for critically reading the manuscript.

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