Cytoplasmic acidification is now recognized as a feature of apoptosis in a variety of systems. However, its relation to other events in the process of apoptosis is not yet characterized. In this work, we examined the effect of BCL-2 overexpression on acidification mediated by cycloheximide treatment or Fas ligation in Jurkat T-lymphoblasts. We find that BCL-2 overexpression attenuates cytoplasmic acidification and apoptosis detected by annexin V labeling. Acidification and phosphatidylserine externalization were found to occur concomitantly. We also examined the requirement for protease activation for cytoplasmic acidification to occur and found that inhibition of interleukin-1β converting enzyme/CED-3 family proteases (using carboxbenzoyl-Val-Ala-Asp-fluoromethylketone, an inhibitor of these proteases) prevents acidification and apoptosis mediated by Fas ligation. These studies suggest that BCL-2 acts at a point upstream of acidification and that protease activation is also upstream of acidification.

Acidification has been described as a feature of programmed cell death in a variety of systems, but its relation to other events in the process is unknown. Engagement of the "death machinery" appears to involve activation of a protease or cascade of proteases belonging to the CED-3/ICE family. Finding that inhibition of interleukin-1β converting enzyme (ICE) family (1, 2) or carboxbenzoyl-Val-Ala-Asp-fluoromethylketone, an inhibitor of these proteases) prevents acidification and apoptosis mediated by Fas ligation. These studies suggest that BCL-2 acts at a point upstream of acidification and that protease activation is also upstream of acidification.

**RESULTS**

BCL-2 Protects Against Acidification and Apoptosis—Expression of BCL-2 has been shown to protect against the induction of apoptosis by cycloheximide (7, 8) and to be partially protective against Fas ligation (4, 5). We examined the effect of BCL-2 expression upon the induction of acidification and phosphatidylserine externalization in the lymphoid cell line, which undergoes apoptosis in response to Fas ligation. Results of flow cytometric pH analysis are shown in Fig. 1A. Upon treatment with anti-Fas antibody, CEM cells stably transfected with the empty vector (CEM/Neo), and when treated with anti-Fas, CEM cells demonstrate acidification and annexin V labeling. In contrast, CEM cells stably transfected with BCL-2 and treated with anti-Fas exhibit less acidification (that is, fewer cells acidify), although the pH change in the cells that do acidify is similar. The resting pH of untreated CEM/Neo and CEM/Bcl-2 cells is similar. The percentage of acidified cells (defined as cells with a 575/620 nm ratio 2 S.D. below the mean of the control population) in the CEM/Neo cell line treated with anti-Fas was 21%, and the percentage of annexin V-labeled cells was 19%. In the CEM/Bcl-2 cells treated with anti-Fas, the percentage of acidified cells was 10%, and the percentage of annexin V-labeled cells was 15%. These results indicate that BCL-2 protects against both acidification and phosphatidylserine externalization. We also found that there was a dose correlation between acidified cells and cells that bound annexin V (Fig. 1A, fourth and fifth panels). In the lymphoid cell line, BCL-2 expression was similar in the two cell lines (Table I). Because BCL-2 expression has been shown to confer protection against apoptosis mediated by cycloheximide, we examined intracellular pH in CEM cells exposed to 100 μg/ml cycloheximide for 3 h (Fig. 1B). As expected, overexpression of BCL-2 protected
against acidification and annexin V labeling. The percentage of acidified cells after cycloheximide was 33.8% for CEM/Neo and 7.7% for CEM/Bcl-2. Annexin V labeled 33.0% of the CEM/Neo (versus 7.0% in CEM/Neo control) and 18.4% of the CEM/Bcl-2 cells (versus 17.5% in CEM/Bcl-2 control).

Role of Protease Activation in Cytoplasmic Acidification—If acidification preceded activation of the death protease cascade, then one would expect acidification to occur in response to Fas ligation even in the presence of the ICE family protease inhibitor peptide, ZVAD. If, however, ICE family protease activation is a prerequisite for acidification to occur, then the peptide should inhibit Fas-mediated acidification. Shown in Fig. 2 are representative flow cytometry plots (pH versus forward scatter) showing the effect of ZVAD pretreatment on pH in cells treated with anti-Fas. Pretreatment of cells with ZVAD prevents acidification mediated by Fas ligation. The lack of acidification parallels the absence of annexin V labeling and DNA fragmentation (data not shown). These findings suggest that activation of CED-3/ICE proteases is an event that is upstream of acidification.

**DISCUSSION**

These studies suggest that pH homeostasis is tightly linked to the control of apoptosis and that both BCL-2 and CED-3/ICE family proteases affect intracellular pH control. These results are consistent with the recent report by Chinnaiyan et al. (10) that BCL-2 functions upstream of the cysteine proteases Yama (CPP32/apopain) and ICE/LAP3 (Mch3). Activation of the protease cascade is considered to be the point of no return in the process of apoptosis. However, we have previously shown that cytoplasmic alkalinization (e.g. by buffering with imidazole) is protective even if done 60 min after Fas ligation (6), by which time protease activation has occurred (11). Taken together, these findings suggest that the irreversible event may be cytoplasmic acidification. Acidification could serve as a global switch to inac-

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**Table I**

| Cell line | 0.01 μg/ml | 0.03 μg/ml | 0.1 μg/ml |
|-----------|------------|------------|-----------|
| CEM/Neo   | 3.18 (1.10)| 3.76 (0.28)| 9.80 (4.25)|
| CEM/Bcl-2 | 2.80 (1.38)| 5.50 (0.81)| 8.05 (0.88)|

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tivate cellular processes that operate at neutral pH and to initiate a previously latent set of enzymes with pH optima below 7.0, such as the acid endonuclease (DNase II) that has been implicated in destruction of the genome (12, 13). Quite possibly, other pH-dependent enzymes could become active when the cell enters a new metabolic state upon acidification.

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**Fig. 2. Effect of ZVAD on acidification mediated by Fas ligation.** CEM/Neo cells were incubated with the ICE inhibitor peptide (ZVAD) for 45 min before the addition of 100 ng/ml anti-Fas IgM. Intracellular pH was assessed by flow cytometry. A representative experiment (one of four) is shown and plotted as forward scatter (y axis) versus pH (x axis); pH 7.0 is indicated.