Bcl-X\textsubscript{L} Cooperatively Associates with the Bap31 Complex in the Endoplasmic Reticulum, Dependent on Procaspase-8 and Ced-4 Adaptor\textsuperscript{*}

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Bap31 is a polytopic integral membrane protein of the endoplasmic reticulum and forms a complex with Bcl-2/Bcl-X\textsubscript{L} and procaspase-8 (Ng, F. W. H., Nguyen, M., Kwan, T., Branton, P. E., Nicholson, W. D., Cromlish, J. A., and Shore, G. C. (1997) J. Cell Biol. 139, 327–338). In co-transfected human cells, procaspase-8 is capable of interacting with Ced-4, an important adaptor molecule in Caenorhabditis elegans that binds to and activates the C. elegans caspase, proCed-3. Here, we show that the predicted death effector homology domain within the cytosolic region of Bap31 interacts with Ced-4 and contributes to recruitment of procaspase-8. Bcl-X\textsubscript{L}, which binds directly but weakly to the polytopic transmembrane region of Bap31, indirectly and cooperatively associates with the Bap31 cytosolic domain, dependent on the presence of procaspase-8 and Ced-4. Ced-4 ac does not interact with Bcl-X\textsubscript{L} but rather displaces it from Bap31, suggesting that an endogenous Ced-4-like adaptor is a normal constituent of the Bap31 complex and is required for stable association of Bcl-X\textsubscript{L} with Bap31 in vivo. These findings indicate that Bap31 is capable of recruiting essential components of a core death regulatory machinery.

Genetic studies in the nematode Caenorhabditis elegans have identified and ordered a core machinery for regulation of apoptotic programmed cell death in which the Bcl-2 homolog, Ced-9, prevents Ced-4 from activating the caspase, Ced-3, and thus blocks ensuing cell death (1–4). Recent reconstitution of these events both in vitro and in heterologous yeast and human cells has revealed that Ced-9 directly binds and sequesters the endogenous Ced-4-like adaptor molecule that otherwise would associate Bcl-X\textsubscript{L} and procaspase-8 (6). Caspase-8 (12–14) belongs to the initiator class of caspases (14–16) whose members appear to function upstream of mitochondria (17) to activate the death pathway. Significantly, Ced-4 itself does not associate with procaspase-3 (6), a downstream effector caspase whose activation may depend on a combination of mitochondrial-released factors and the Ced-4-like cytosolic protein, Apaf-1 (Ref. 18 and reviewed in Ref. 19). A major question, however, is the mechanism by which these Ced-4-controlled events are linked to the plethora of signals that result in activation of caspases and subsequent cell death.

Recently, we identified a Bcl-2/Bcl-X\textsubscript{L} and procaspase-8 associated protein in the endoplasmic reticulum (ER),\textsuperscript{1} p28 Bap31 (20). Bcl-2 family proteins are located in the ER/nuclear envelope and mitochondrial outer membrane (21–23) and, in the latter location, appear to prevent activation of downstream effector caspases such as procaspase-3 in response to diverse death signals (see Refs. 19 and 24). Initiator caspases such as procaspase-8, on the other hand, are well characterized constituents of the Fas and TNFR1 apoptosis signaling complexes in the plasma membrane (25–27). These complexes, however, are highly restricted in the death signals to which they respond and are not directly influenced by Bcl-2 family proteins. The ability of Bap31 to associate with both procaspase-8 and Bcl-2/Bcl-X\textsubscript{L}, therefore, raises the possibility that the Bap31 complex in the ER might cooperate with events in the mitochondrion to control proximal and distal steps in a Bcl-2-regulated caspase cascade. If so, the ability of Ced-4 to bridge Bcl-X\textsubscript{L} and procaspase-8 (6) predicts that a Ced-4-like adaptor molecule may also be a part of the Bap31 complex.

EXPERIMENTAL PROCEDURES

Plasmids—cDNAs encoding proteins tagged with specific epitopes were constructed in expression vectors. Flag epitope was inserted toward the C terminus of Bap31, immediately upstream of the KEEK ER envelope and mitochondrial outer membrane (25–27). These complexes, however, are highly restricted in the death signals to which they respond and are not directly influenced by Bcl-2 family proteins. The ability of Bap31 to associate with both procaspase-8 and Bcl-2/Bcl-X\textsubscript{L}, therefore, raises the possibility that the Bap31 complex in the ER might cooperate with events in the mitochondrion to control proximal and distal steps in a Bcl-2-regulated caspase cascade. If so, the ability of Ced-4 to bridge Bcl-X\textsubscript{L} and procaspase-8 (6) predicts that a Ced-4-like adaptor molecule may also be a part of the Bap31 complex.

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1 The abbreviations used are: ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; HA, hemagglutinin.

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RESULTS AND DISCUSSION

The predicted death effector homology domain in Bap31 (amino acids 163–238) is flanked on either side by sites that are cleaved by caspase-8 or a related caspase during adenovirus E1A-induced apoptosis (20) (summarized in Fig. 1A). The resulting p20 Bap31 product is a potent inducer of apoptosis when expressed ectopically in otherwise normal cells, presumably because it has a dominant-negative effect on endogenous Bap31 (20). In vitro mapping has revealed that Bcl-2 proteins bind directly to the N-terminal domain of Bap31, which includes the polytopic membrane-associated region, and weakly if at all with the cytosolic domain (20). As expected, therefore, full-length Flag-tagged Bap31, but not the Bap31 cytosolic domain alone (Bap31ΔN119), interacted with Bcl-XL in cotransfected human 293T cells, as judged by co-immunoprecipitation with anti-Flag antibody (Fig. 1B, compare lanes 3 and 4). However, when Bap31ΔN119 was coexpressed with both Bcl-XL and procaspase-8 (proFLICE), a strong association between Bcl-XL and the Bap31 cytosolic domain was recorded (lane 5). Thus, Bcl-XL may be tethered at the N terminus of Bap31 and associate indirectly and cooperatively with the Bap31 cytosolic domain, dependent on the presence of caspase-8. Failure to achieve reconstitution of these interactions in vitro (not shown), however, suggested that an adaptor molecule might be required.

As a preliminary step indicating that Ced-4 might associate with Bap31, a transcription-translation product of the proapoptotic short splice form of Ced-4 (28) was found to interact in vitro with a glutathione S-transferase fusion protein containing the Bap31 cytosolic domain (GST-Bap31ΔN119) (Fig. 2). Furthermore, when Ced-4 was coexpressed with Bap31-Flag in 293T cells and total cell extracts were incubated with anti-Flag antibody, Ced-4 co-immunoprecipitated with Bap31-Flag but not with Control-Flag (Fig. 3A, lanes 3 and 4). This association was significantly reduced using a Bap31 construct lacking the putative death effector homology domain (Bap31Δ167–240-Flag, lane 2). In fact, the residual amount of Ced-4 that was recovered in association with the Bap31 deletion mutant (lane 2) may have resulted because immunoprecipitation of Bap31Δ167–240-Flag with anti-Flag antibody also precipitated endogenous Bap31 (lanes 7). The latter is consistent with our findings that Bap31 forms homooligomers both in vitro and in vivo. Of note, expression of Ced-4 did not lead to significant caspase-dependent cleavage of Bap31. This is concordant with the observation that Ced-4 does not independently induce apoptosis in 293T cells (9).

Importantly, the presence of Bcl-XL did not influence the ability of Ced-4 to associate with Bap31 in 293T cells (Fig. 3A, lanes 4 and 6), suggesting that Bcl-XL does not function to prevent Ced-4 from engaging the Bap31 complex. Moreover, this was also extended to procaspase-8, where it was found that Ced-4 and procaspase-8, when expressed individually in cotransfected 293T cells, associated with Bap31-Flag (Fig. 3B, lanes 3 and 5) to the same extent as in the situation where Ced-4 and procaspase-8 were expressed together (lane 7). Again, as was the case for Ced-4 (Fig. 3A), the ability of procaspase-8 to associate with Bap31 was significantly reduced by

\[ ^2 \text{F. W. H. Ng and G. C. Shore, unpublished observation.} \]
deleting the putative Bap31 death effector homology domain (Fig. 3C).

One obvious explanation to account for the ability of Ced-4 to associate with the Bap31 complex is that Ced-4 may be substituting for an endogenous Ced-4-like adaptor molecule. Earlier studies identified a means of testing this hypothesis (Ref. 6, see schematic in Fig. 4). A mutant Ced-4 lacking the C-terminal 248 amino acids, Ced-4Dc, failed to interact with Ced-9 or Bcl-XL but retained the ability to associate with Ced-3 or pro-caspase-8 in co-transfected 293T cells. Thus, it competes in vivo with an endogenous adaptor that bridges Bcl-XL and pro-caspase-8 and prevents precipitation of procaspase-8 by an antibody directed to Bcl-XL (6). Similarly, we found that Ced-4Dc lacking the C-terminal 246 amino acids also competed for the ability of Bcl-XL to bind to Bap31 in vivo (Fig. 4, lanes 1 and 3, upper panel), strongly implying that it does so by displacing an endogenous adaptor that otherwise would make contact with Bcl-XL. Wild-type Ced-4 also reduced somewhat the level of Bcl-XL that was recovered with Bap31 (lanes 1 and 2), suggesting that Ced-4 is less efficient than the endogenous adaptor molecule at contributing to the association of Bcl-XL with the Bap31 complex. Taken together, the results in Fig. 4 and those in Fig. 1B demonstrate that cooperative interactions between Bcl-XL and both Ced-4 and procaspase-8 contribute to the stable binding of Bcl-XL to Bap31 in vivo.

In conclusion, the ability of Ced-9 to bind to and prevent Ced-4 from activating Ced-3 provides a simple explanation to account for the molecular control over apoptotic cell death. Nevertheless, it remains to be explained how the corresponding molecular complexes in mammalian cells are linked to the multitude of signals that can trigger activation of procaspases and how they interface with the numerous proapoptotic and antiapoptotic regulators that modulate these signals (24, 29). The cooperative associations between Bcl-XL, Ced-4, pro-caspase-8, and the Bap31 cytosolic domain observed here, on the other hand, suggest that Ced-4 is a normal constituent of the Bap31 complex.

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