Supplementary Material

*Mutations in the BH3-binding pocket of Bcl-x<sub>L</sub> correlate dimer formation with mitochondrial import and bioactivity*

Previously, G138A was found to block Bcl-x<sub>L</sub> binding to Bax in the presence of detergent and to inhibit Bcl-x<sub>L</sub> bioactivity (Sedlak et al., 1995). Confirming the prior report, this mutant was substantially deficient in anti-apoptotic activity (Supplemental Figure 1A) and Bax binding (data not shown). We found that this mutation also reduced Bcl-x<sub>L</sub> homodimer formation in the absence of detergents (Supplemental Figure 1B). The Y101K mutant of Bcl-x<sub>L</sub> was reported to prevent Bcl-x<sub>L</sub> binding to Bax in the presence of detergent but not to inhibit Bcl-x<sub>L</sub> bioactivity (Minn et al., 1999). Unlike G138A, this mutation does not inhibit Bcl-x<sub>L</sub> homodimerization (Supplemental Figure 1B) and retains bioactivity (Supplemental Figure 1A). There was no dramatic difference in folding between wt, Y101K and G138A Bcl-x<sub>L</sub> as detected by limiting proteolysis analysis (data not shown). Thus, in contrast to their ability to heterodimerize with Bax, the ability of the Bcl-x<sub>L</sub> mutants in the BH3-binding pocket to homodimerize correlates with bioactivity. However, although both Y101K and G138A failed to bind Bax both were able to bind Bad (data not shown). This extends the correlation between homodimer formation and bioactivity of Bcl-x<sub>L</sub> seen with the C-tail mutations in Figure 3. How does homodimer formation contribute to the bioactivity of Bcl-x<sub>L</sub>? As mitochondrial binding of Bcl-x<sub>L</sub> appears essential for bioactivity, we examined the cell free import of BH3-binding pocket mutants of Bcl-x<sub>L</sub> into mitochondria. The G138A
mutant reduced to form dimers, lacked bioactivity, and showed less import into mitochondria whereas the Y101K mutant retained dimer formation, bioactivity, and imported into mitochondria as well as wt Bcl-x<sub>L</sub> (Supplemental Figure 1C). All three of these Bcl-x<sub>L</sub> proteins have the same C-tail sequence that would be predicted to bind mitochondria identically (Figure 3E, Kaufmann et al., 2003). Thus, Bcl-x<sub>L</sub> homodimer formation correlates with mitochondrial import competence and may be important for this process (Table I).

**Supplemental Figure 1.** Mutations at G138 and Y101 in the BH3-binding pocket of Bcl-x<sub>L</sub> correlate dimer formation with mitochondrial import and bioactivity. (A) Viability assays of wt Bcl-x<sub>L</sub> and Bcl-x<sub>L</sub> mutants. Jurkat cells were co-transfected with YFP-Bcl-x<sub>L</sub> constructs and Bax (ratio, 1:2) and incubated for 12 h and treated with 100 µM etoposide for 6 h. The viability assay experiment was performed as described in Figure 3C. (B) Co-IP of C-terminal mutants of myc-Bcl-x<sub>L</sub> and Bcl-x<sub>L</sub>. Cos-7 cells were co-transfected with each mutant of myc-Bcl-x<sub>L</sub> and Bcl-x<sub>L</sub>, as indicated in the figure (ratio, 1:1), and myc-Bcl-x<sub>L</sub> was immunoprecipitated from the cytosol fraction (S-100) of cells by anti-myc antibody. The immunoprecipitated and the input samples were analyzed by Western blotting with anti-Bcl-x<sub>L</sub> 4C3 antibodies. (C) G138A mutant does not import into mitochondria in vitro. Radiolabeled wt Bcl-x<sub>L</sub>, Y101K, and G138A were incubated separately with mitochondria isolated from early stage apoptotic HeLa cells for 15 min at 37°C and the import experiment was performed as described in Figure 3D.
Supplemental Figure 1.