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COMMENT

Avoiding adsorption of Bcl-2 proteins to plasticware is important for accurate quantitation

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Recombinant Bcl-2 proteins are used in mitochondrial and liposomal assays to gain insights into their regulation of the mitochondrial (intrinsic) pathway of apoptosis. While quantitating Bcl-2 protein function in mitochondrial assays we found the major loss of recombinant Bcl-2 proteins when diluting from micromolar to low nanomolar concentrations. In particular, when solutions of Bid, Bim, Bax or Bcl-x_L were diluted, most protein bound to the plasticware (i.e., tubes and pipette tips). Adsorption of these recombinant proteins was greatly diminished by the simple addition of 1% bovine serum albumin (BSA) to dilution buffers. Published studies have not addressed this non-specific binding of Bcl-2 proteins to surfaces, thus it is likely the potency of Bcl-2 proteins has been underestimated in many in vitro studies.

It is well known that proteins can adsorb to various solid surfaces through hydrophobic, electrostatic or other interactions [1–6]. Indeed, proteins with a low internal stability, such as BSA, can adsorb on all surfaces owing to a gain in conformational entropy resulting from adsorption [7]. Strategies to mitigate protein adsorption include supplementing with BSA [8] or nonionic detergents [9], or coating surfaces with proteins or pegylation reagents [8, 10]. As these strategies are absent from the methods sections of most (if not all) studies of the Bcl-2 family members, the potency of these critical apoptosis regulators may have been significantly underestimated.

To test for adsorption of Bcl-2 proteins we examined the effect of supplementing the dilution buffer with 1% BSA for six recombinant Bcl-2 family members (Fig. 1a). The dilution buffer comprised a standard Tris-buffered saline (TBS; 20 mM Tris pH 8.0, 150 mM NaCl) with or without 1% BSA supplementation. Initial Bcl-2 protein concentrations were in the micromolar range (19–533 μM) and diluted to 5 μM, then to 0.5 μM prior to final concentrations of 0.05 μM. When tested by western blot, the final protein concentrations were significantly higher if 1% BSA was present in the dilution buffer. BSA appeared to fully counter adsorption as Bcl-2 protein levels after dilution in BSA were equivalent to those achieved by dilution in SDS gel loading buffer (Fig. 1a). The hydrophobic C-terminal transmembrane domains present in many Bcl-2 family members were not solely responsible for adsorption, as BSA had a similar effect on proteins lacking the membrane-binding C-terminus ($Bim_S\Delta C$, Bcl-$x_L\Delta C$, and $Bax\Delta C$) (Fig. 1a).

The presence of BSA during dilution greatly enhanced the estimated potency of Bid in mitochondrial cytochrome *c* release assays (Fig. 1b). In these experiments, recombinant cleaved Bid or a series of Bid BH3 chimeras were incubated with isolated mouse liver mitochondria and cytochrome *c* release measured, as previously [11]. The EC_{50} for each chimera was estimated using % cytochrome *c* release initiated by a range of chimera concentrations. In three independent experiments Bid proteins that were diluted in the presence of 1% BSA showed significantly higher potency (lower EC_{50}), consistent with higher Bid levels present following dilution in BSA (Fig. 1a). The increase in potency (10-30 fold) following dilution in the presence of BSA indicates >90% loss of the protein during dilution in buffer only. Further addition of BSA to the mitochondrial incubation itself did not enhance cytochrome *c* release induced by Bid (not shown).

Preventing the adsorption of Bcl-2 family proteins to surfaces (e.g., polypropylene tubes) when diluting recombinant protein preparations is thus important in quantitative

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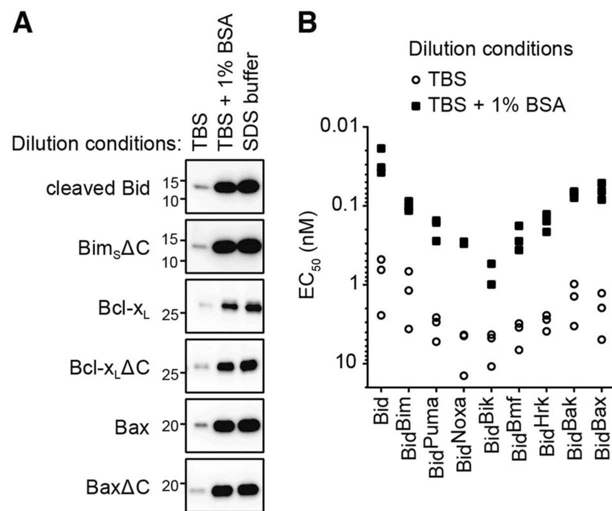

Fig. 1 Loss of Bcl-2 family proteins during dilution is rescued by addition of BSA to the diluent. **a** Dilution of recombinant Bcl-2 family proteins in the absence of BSA results in major loss of protein. The indicated Bcl-2 proteins were diluted in three steps to obtain 5 µM and 0.5 µM and then to 0.05 µM final concentrations. Diluents were TBS (20 mM Tris pH 8.0, 150 mM NaCl), TBS with 1% (w/v) BSA, or SDS gel-loading buffer (85 mM Tris, 75 mM NaCl, 0.6% SDS, 15% glycerol, 9 µg/ml bromophenol blue, 2.5% β-mercaptoethanol). Tubes were polypropylene 0.5 ml PCR tubes (Axygen PCR-05-C) or 1.5 ml tubes (Eppendorf, 0030120.085). Aliquots of the final 0.05 µM solutions were taken for western blot (and after equalizing the buffer conditions for the three samples by addition of appropriate BSA and/or SDS gel-loading buffers). Membranes were probed with rat monoclonal antibodies for Bid (clone 2D1), Bim (clone 3C5), Bcl-x_L (clone 9C9) or Bax (clone 49F9) generated by WEHI Antibody Facility, Bundoora, Australia. Recombinant human Bcl-2 proteins (28 µM caspase-8 cleaved Bid; 278 µM Bim_SΔC27; 19 µM Bcl-x_L; 71 µM Bcl-x_LΔC25; 146 µM Bax; and 533 µM BaxΔC21) were generated as described previously [11, 12]. Blots are representative of two or more independent experiments. **b** Dilution of Bid chimeras in the absence of BSA results in 30-fold lower cytochrome *c* release from mitochondria. The indicated Bid and Bid chimeras [11] were diluted in TBS with or without 1% BSA to generate stocks of 1 µM, 0.1 µM, 0.01 µM and 0.001 µM and then added (at 1:100 or 3:100 dilution) to isolated mouse liver mitochondria. Following incubation, cytochrome *c* release was measured by western blot, and the potency (EC_{50}) of each reagent estimated as the dose that caused 50% cytochrome *c* release (see [11]). Experiments with TBS + 1% BSA were published previously [11]. EC_{50} estimates from three independent experiments are shown

studies such as measuring the potency of proteins and mathematical modelling [11]. Adsorption may be less relevant in experiments designed to ascertain pro- or anti-apoptotic function, especially if high protein concentrations are used. As BSA was effective in minimizing adsorption of several Bcl-2-related proteins (14 tested here) during a 3-step dilution series, supplementation of dilution buffers with BSA provides a simple means to more accurately test the potency of Bcl-2 family proteins in vitro.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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