RESEARCH COMMUNICATION

The study of cell-death proteins in the outer mitochondrial membrane by chemical cross-linking

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Chemical cross-linking was used to study the interactions of the anti-cell-death protein Bcl2 with other proteins in the outer mitochondrial membrane. Cross-linking of mitochondrial surface proteins produced a large Bcl2-containing complex (> 200 kDa),

and a Bcl2-derived peptide was shown to cross-link specifically with a mitochondrial protein identified by immunoblotting as Raf-1 kinase.

INTRODUCTION

In recent years the molecular basis of cell-death regulation has come under intense investigation [1,2]. Particular emphasis has been placed on the *bcl2* gene and its product, which was originally cloned due to its proximity to a t(14:18) translocation breakpoint implicated in B-cell lymphomas [3,4]. Subsequent analysis revealed that over-production of the Bcl2 protein results in the promotion of cell survival, even in the presence of toxic insults that normally cause cell death. The function of the Bcl2 protein is to maintain long-lived post-mitotic cells, such as memory B-cells, in the thymus. Related proteins have since been identified with either pro- or anti-cell-death activities [1,3].

Subcellular localization of the Bcl2 protein has revealed that it interacts primarily with the outer mitochondrial membrane [5], and topology studies indicate that Bcl2 is inserted into the membrane via its C-terminal hydrophobic domain such that residues 1–210 (encoding the functionally important domains) protrude out into the cytosol [6]. The solving of the three-dimensional structure of the closely related Bcl-X protein has provided some insight into the biochemical function of these proteins [7] by revealing that soluble Bcl-X (lacking the membrane-anchor domain) consists of an N-terminal α -helical region joined via a flexible linker to a globular domain with a fold reminiscent of diphtheria toxin, suggestive of an ion channel. Further studies have shown that Bcl-X can form cation-specific channels in planar membranes and vesicles, although the physiological role these channels play in preventing cell death remains unknown [8].

In an attempt to understand further the biochemical mechanism by which Bcl2 functions in its target membranes, and in order to detect complex or transient protein–protein interactions, we have adopted a novel approach of using chemical cross-linking agents upon isolated mitochondria to identify proteins that interact with Bcl2.

MATERIALS AND METHODS

The Bcl2-overproducing lymphoblastoid cell line RS11846 and the control lymphoblastoid cell line AG110 were grown in

RPMI1640 medium supplemented with 10 % (v/v) fetal bovine serum, 2 mM L-glutamine, 100 units·ml^{−1} penicillin and 100 μ g ml^{−1} streptomycin and were grown to a final density of 1×10^8 cells·ml^{−1}. Cells were harvested by centrifugation (5000 g, 10 min, 4 °C), and the pellets were washed twice with $1 \times$ PBS. Mitochondria were isolated from both tissue-culture cells and tissue samples by homogenization in 5 vol. of buffer (0.3 M sucrose, 1 mM EGTA, 5 mM Mops, 5 mM KH₂PO₄, pH 7.4) using a Teflon homogenizer, followed by a series of low-speed (1000 g, 10 min, 4 °C) and high-speed (10000 g, 10 min, 4 °C) centrifugations to remove whole cells and debris [9]. Crude mitochondrial pellets were then subjected to isopycnic centrifugation on a 1–2 M sucrose gradient. Mitochondrial purity was confirmed by marker enzyme assays and were stored frozen in homogenization buffer until required.

Mitochondria were thawed on ice and washed twice with cross-linking buffer (20 mM sodium phosphate, pH 7.2) before being resuspended in the same buffer at a total protein concentration of 1 mg·ml^{−1}. Chemical cross-linkers were purchased from Pierce Chemical (Rockford, IL, U.S.A.), prepared as 100 mM stock solutions in DMSO and stored frozen. The cross-linkers were used according to the manufacturer's instructions.

In a typical cross-linking experiment, 100 μ g of total mitochondrial protein was incubated with 1 μ g of peptide for 2 h in an ice bath, after which time 1 μ l of chemical cross-linking reagent was added (final concentration 1 mM), and the reaction was left to proceed at room temperature for 30 min. Excess cross-linking reagent was quenched by the addition of 50 mM Tris/glycine buffer, pH 7.0, and leaving for 10 min at room temperature. For photoactivatable cross-linking reagents, the reaction was performed in two steps. Cross-linking reagent (stored in the dark) was added to the reaction mix under safelight conditions, and the reaction was allowed to proceed in the dark for 30 min. Unreacted N-hydroxysuccinimide esters were quenched by adding 50 mM Tris/glycine buffer and leaving in the dark for a further 10 min. Mitochondria were then pelleted and resuspended in fresh cross-linking buffer (if required) before photoactivation of the cross-linking reagent by exposure to UV light (302 nm) for 15 min.

Abbreviations used: sulpho-SBED, 2-[6-(biotinamido)-2-(p-azidobenzamido)-hexanoamido]ethyl-1,3'-dithiopropionate; sulpho-HSAB, N-hydroxysulphosuccinimidyl-4-azidobenzoate; DSS, disuccinimidyl suberate; EGS, ethylene glycol-bis(succinimidylsuccinate); mp80, mitochondrial protein 80 kDa.

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Cross-linked products were resuspended in SDS loading buffer, separated on an SDS/8%-polyacrylamide gel, and transferred to PVDF membrane (Amersham, Amersham, Bucks., U.K.) in 10 mM Caps buffer, pH 11.0, containing 10% (v/v) methanol [10]. Membranes were incubated with primary antibodies for Bcl2 peptide N19 or Raf-1 kinase at 1:750 dilution for 18 h or at 1:500 dilution for 4 h. A horseradish peroxidase-conjugated secondary antibody was used at 1:2000 dilution (1 h), and immunoreacted bands were revealed using the enhanced chemiluminescence detection kit (Amersham). All antibodies and their corresponding peptides were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), and immunoprecipitation experiments were performed using the protocol provided. Prestained molecular-mass markers were purchased from Sigma (St. Louis, MO, U.S.A.).

RESULTS AND DISCUSSION

To test for the existence of Bcl2 partner proteins in the outer mitochondrial membrane, mitochondria were isolated from the human lymphoblastoid tumour cell line RS11846, which overproduces the Bcl2 protein due to the presence of the t(14:18) translocation. Mitochondria were resuspended in a cross-linking buffer at a concentration of $1 \text{ mg} \cdot \text{ml}^{-1}$, and surface proteins were cross-linked by incubating with the membrane-impermeable,

biotinylated, heterobifunctional cross-linker 2-[6-(biotinamido)-2-(*p*-azidobenzamido)-hexanoamido]ethyl-1,3'-dithiopropionate (sulpho-SBED) or with *N*-hydroxysulphosuccinimidyl-4-azidobenzoate (sulpho-HSAB) (Figure 1a). Analysis of the cross-linked products by immunoblotting, using an anti-Bcl2 antibody, revealed a single high-molecular-mass product greater than 211 kDa in size (Figure 2, lane 8). Further analysis of the same blot using streptavidin-horseradish peroxidase to detect all cross-linked products (via the biotin moiety in the sulpho-SBED cross-linker) showed the presence of six complexes of varying molecular mass (results not shown). This indicates that Bcl2 is specifically cross-linked to a particular target protein(s). Attempts to resolve and characterize this high-molecular-mass complex further were hindered by its failure to enter low-percentage acrylamide gels. A series of heterobifunctional cross-linkers of varying length (9.0–18.5 Å) were then employed to ascertain whether a more specific Bcl2-containing cross-linked product of lower molecular mass could be identified on the surface of RS11846 mitochondria. Again the only cross-linked product detected using anti-Bcl2 antibody had a molecular mass greater than 211 kDa. Furthermore, no cross-linking was observed under these conditions when cross-linkers of less than 13.9 Å in length were used (sulpho-HSAB, 9.0 Å; Figure 2, lanes 3–5).

The Bcl2 protein sequence contains four lysine residues, which in addition to the N-terminal amino group are the only targets

Figure 1 Cross-linking reagents and peptides used in this study

(a) The photoactivatable (aryl-nitrene group) and amine-reactive (*N*-hydroxysuccinimide ester) heterobifunctional cross-linkers sulpho-HSAB (top) and sulpho-SBED (bottom) are shown. The photoreactive group can modify a number of different amino acid side chains, and presence of the sulphite ion prevents membrane permeability. The biotinylated cross-linker sulpho-SBED also contains a cleavable disulphide, permitting separation of the cross-linked products. (b) The amine-reactive homobifunctional cross-linkers disuccinimidyl suberate (top) and ethylene glycol-bis(succinimidylsuccinate) (bottom) are shown. Both are membrane permeable, but only ethylene glycol-bis(succinimidylsuccinate) can be cleaved with hydroxylamine. (c) The N-terminal sequence of human Bcl2 is presented with the regions corresponding to peptides N19 (residues 4–21) and N30 (residues 4–33) indicated above the sequence.

Figure 2 Chemical cross-linking of surface proteins from intact mitochondria

Mitochondria (100 μ g of total protein) isolated from the Bcl2-overproducing cell line RS11846 were mixed with 0.01–1 mM heterobifunctional photoactivatable cross-linking reagent, incubated in the dark for 2 h, washed to remove unreacted cross-linker, and photoactivated with UV light for 15 min to complete the cross-linking reaction. Cross-linked products were separated on an SDS/12%-polyacrylamide gel and revealed by immunoblotting. Lane 1, control; lane 2, DMSO; lane 3, 0.01 mM sulpho-HSAB; lane 4, 0.1 mM sulpho-HSAB; lane 5, 1 mM sulpho-HSAB; lane 6, 0.01 mM sulpho-SBED; lane 7, 0.1 mM sulpho-SBED; lane 8, 1 mM sulpho-SBED. Positions of molecular-mass markers are shown on the left. Arrow indicates position of Bcl2-containing cross-linked protein complex.

Figure 3 Chemical cross-linking of peptide N19 to a specific mitochondrial surface protein

Mitochondria (100 μ g of total protein) isolated from the control lymphoblastoid cell line AG110 were incubated on ice with 1 μ g of peptide N19 for 2 h before the addition of the homobifunctional cross-linker DSS (1 mM). Cross-linked products were separated on an SDS/12%-polyacrylamide gel and revealed by immunoblotting. Lane 1, peptide N19 alone; lane 2, DSS alone; lane 3, peptide N19 cross-linked to proteins with DSS; lane 4, as lane 3 except that mitochondria were washed once in cross-linking buffer before the addition of DSS. Positions of molecular-mass markers are shown on the left. Arrow indicates position of cross-linked peptide-protein complex.

for the amine-reactive N-hydroxysuccinimide ester that comprises one half of the reactive groups on the cross-linkers used. Interestingly, the four lysine residues are distributed such that two are located in the conserved N-terminal region of Bcl2 (Lys¹⁷ and Lys²²) and two are located in the C-terminal transmembrane region (Lys²¹⁸ and Lys²³⁹). As the cross-linkers used in the initial study are all membrane impermeable, the N-terminal amino group plus Lys¹⁷ and Lys²² are the likely sites of modification. A synthetic peptide corresponding to residues 4–21 of Bcl2 (Bcl2

peptide N19; Figure 1c) and an antibody that recognizes it were purchased from Santa Cruz Biotechnology. Peptide N19 (1 μ g) was incubated with intact mitochondria isolated from the human control lymphoblastoid cell line AG110 (which does not over-produce Bcl2) and cross-linked. Analysis by immunoblotting revealed that all the cleavable cross-linkers tested (9.0–18.5 Å) covalently attached peptide N19 to a protein when the samples were separated on a non-reducing SDS/polyacrylamide gel (results not shown).

The protein to which peptide N19 preferentially bound was further characterized using the non-cleavable homobifunctional cross-linker disuccinimidyl suberate (DSS), which has a linker region of 11.4 Å with an amine-reactive N-hydroxysuccinimide group at either end (Figure 1b). Cross-linking of peptide N19 to intact AG110 mitochondria followed by immunoblotting revealed that a protein of approx. 80 kDa [mitochondrial protein 80 kDa (mp80)] was the target for binding by the N-terminal region of Bcl2 (Figure 3, lane 3). Washing of the mitochondria before adding the cross-linker decreased the amount of cross-linked product detected, but did not eliminate it (Figure 3, lane 4). This suggested that peptide N19 strongly recognizes mp80. To confirm that the observed peptide–protein cross-linking did not arise from non-specific binding, a series of cross-linking experiments were performed using peptides derived from the N-termini of the pro-cell-death protein Bax (peptide N-20; residues 11–30) and c-Fos [peptide c-Fos(4); residues 3–16]. Cross-linked products were analysed by Western blotting using the corresponding antibodies (results not shown). Only Bcl2 peptide N19 specifically cross-linked mp80 and could be detected by anti-N19 antibody.

The specificity of the interaction between peptide N19 and mp80 was further confirmed using mitochondria isolated from mouse brain. Mitochondria, at a total protein concentration of 1 mg·ml⁻¹, were incubated with increasing quantities of peptide N19 (0–1000 ng) and cross-linked with DSS. Analysis by immunoblotting confirmed that a single protein is preferentially modified, with some binding of peptide N19 occurring at 50 ng and saturation of mp80 sites being complete by 100 ng of peptide (results not shown). The distribution of mp80 in mammalian tissues was also examined. Mitochondria isolated from mouse brain, heart and liver were analysed for peptide binding by the cross-linking assay. Cross-linking of peptide N19 to an 80 kDa protein was observed to occur in mitochondria from all three tissues (results not shown).

The data presented here indicate that the conserved N-terminal region of Bcl2 interacts directly with an 80 kDa outer mitochondrial membrane protein in a highly specific manner. The protein, mp80, appears to have a wide tissue and species distribution, which is consistent with it being a conserved housekeeping protein. The ability of Bcl2 to interact with, and possibly modify the function of, such a protein would be expected, since it has been observed that Bcl2 can impede the death of cells in which it is not normally produced [3].

Attempts to identify mp80 by protein microsequencing of material enriched by either immunoprecipitation of the peptide–protein complex or affinity chromatography both indicated that the N-terminus was blocked. A number of candidates were considered, including the heat-shock protein GRP78 and protein kinase C ϵ ; however, none was found to be mp80. Recently, Raf-1 kinase has been implicated in the regulation of the cell-death pathway, and the N-terminal region of Bcl2 is believed to be responsible for recruiting Raf-1 kinase to the mitochondrial membrane [11–13]. To test whether mp80 is Raf-1 kinase, immunoblotting studies were carried out in which mp80 was cross-linked to Bcl2 peptide N19 and then detected with both anti-N19 antibody and an anti-Raf-1 kinase antibody that

Figure 4 mp80 is the protein Raf-1 kinase

Mouse-brain mitochondria (100 μ g of total protein) were cross-linked to peptide N19 (1 μ g) with the cross-linker DSS, and the sample was immunoblotted in triplicate. The membrane filter was cut to separate the lanes, and each strip was incubated with a different antibody. Lane 1, anti-Bcl2 N19 antibody; lane 2, anti-Raf-1 kinase (C12) antibody; lane 3, anti-Bcl2 N19 antibody plus anti-Raf-1 kinase (C12) antibody. Positions of molecular-mass markers are shown on the left and right.

recognizes residues 637–648 of the mammalian protein. Figure 4 shows that mp80 and Raf-1 kinase do indeed co-migrate (lanes 1 and 2). Incubation of the blot with both antibodies confirmed that the same protein is recognized by both antibodies and that therefore the 80 kDa protein detected by cross-linking with peptide N19 is Raf-1 kinase (Figure 4, lane 3). A second anti-Raf-1 kinase antibody (C-20, residues 629–648) also gave the same result (results not shown). The predicted mass of Raf-1 kinase is 74–76 kDa by SDS/PAGE, which is in reasonable agreement with a value of 80 kDa obtained here after covalent modification of the Raf-1 kinase with peptide N19 (2.2 kDa).

These chemical cross-linking studies were performed to establish whether the Bcl2 protein interacted directly with any proteins in the outer mitochondrial membrane, with the intention of further defining the role mitochondria play in regulating programmed cell death at the molecular level. The data presented here demonstrate that Bcl2 interacts specifically with Raf-1 kinase in the outer mitochondrial membrane via its N-terminal conserved region.

Earlier attempts to resolve the high-molecular-mass complex obtained during the initial cross-linking studies (Figure 2, lane 8) did not succeed, and so experiments to ascertain whether other proteins might also interact with the N19–Raf-1 kinase complex were carried out, using the homobifunctional cross-linkers DSS (11.4 Å) and ethylene glycol-bis(succinimidylsuccinate) (EGS) (16.0 Å). No increase in the molecular mass of the N19–Raf-1 kinase complex was observed with DSS as expected; however, upon analysis by immunoblotting EGS appears to cross-link peptide N19 into a high-molecular-mass complex (> 211 kDa), as has been previously observed with other cross-linkers (Figure 2). The composition of the Bcl2-containing high-molecular-mass complex remains to be fully elucidated, although preliminary evidence from cross-linking studies using the homobifunctional cross-linker EGS suggests that in addition to Bcl2, the pro-cell-death protein Bad [14] and Raf-1 kinase are likely components (results not shown). Raf-1 kinase can also form a high-molecular-mass complex of 300–500 kDa with the molecular chaperones hsp90 and p50 in the cytosol [15], and this Raf-1 kinase–hsp90–p50 complex translocates from the cytosol to the plasma membrane, where it interacts with Ras and mitogen-activated-

protein kinase kinase. Similarly, Bcl2 could recruit the same complex to the outer mitochondrial membrane, where it could interact with members of the Bcl2 family [3] and R-Ras [16]. To elucidate further the nature of the Bcl2-containing protein complex, a family of homobifunctional arginine-reactive cross-linkers are presently being synthesized with the intention of cross-linking Bcl2 with other proteins in the mitochondrial membrane through internal arginine residues, particularly Arg¹⁴³, which is conserved in all members of the Bcl2 family of cell-death regulators [3,4].

The peptide–protein interaction described between peptide N19 and Raf-1 kinase is highly specific. To understand the specific nature of the binding, a 30 residue peptide (N30; Figure 1c; Thistle Peptides, Glasgow, U.K.) corresponding to residues 4–33 of the human Bcl2 sequence was subjected to a preliminary structural analysis by CD and was found to have a substantial helical content (N. C. Price, S. M. Kelly and S. T. Ali, unpublished work). Recently, a detailed analysis of the N-terminal region using site-directed mutagenesis and synthetic peptides plus CD has identified five hydrophobic and aromatic residues as being necessary for function [17], of which four lie within the region encoded by peptide N19. The region encompassing these five residues can adopt an α -helical conformation in detergent micelles, and the three-dimensional structure for the Bcl2-related protein Bcl-X also shows that the N-terminal region of Bcl-X folds as an α -helix [7]. It is possible that this ability of the N-terminal region of Bcl2 to adopt an α -helical conformation may account for its facility to bind tightly to the surface of Raf-1 kinase to permit specific cross-linking.

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