

Tissue distribution of Diablo/Smac revealed by monoclonal antibodies

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Abstract

Diablo/Smac is a mammalian pro-apoptotic protein that can antagonize the inhibitor of apoptosis proteins (IAPs). We have produced monoclonal antibodies specific for Diablo and have used these to examine its tissue distribution and subcellular localization in healthy and apoptotic cells. Diablo could be detected in a wide range of mouse tissues including liver, kidney, lung, intestine, pancreas and testes by Western blot analysis. Immunohistochemical analysis found Diablo to be most abundant in the germinal cells of the testes, the parenchymal cells of the liver and the tubule cells of the kidney. In support of previous subcellular localization analysis, Diablo was present within the mitochondria of healthy cells, but released into the cytosol following the induction of apoptosis by UV.

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Keywords: Diablo; Smac; apoptosis; tissue; distribution; antibodies

Abbreviations: mAbs, monoclonal antibodies; IAPs, inhibitor of apoptosis proteins; BIR, baculoviral IAP repeat; s.c., subcutaneous; i.p., intraperitoneally; i.v., intravenously; FITC, fluorescein-isothiocyanate; HA, epitope (YPYDVPDYA) tag; HA Diablo, epitope HA-tagged Diablo protein; IP, immunoprecipitation; IF, immunofluorescence; WB, Western blotting; FACS, fluorescence-activated cell sorter

Introduction

Apoptosis in insects and vertebrates can be controlled by inhibitor of apoptosis proteins (IAPs) that act by directly binding to activated caspases. In *Drosophila*, the IAPs (DIAP1 and DIAP2) are antagonized by three pro-apoptotic proteins, Grim, HID and Reaper, which bind to them, thereby displacing the active caspases.^{1–4} In mammals, Diablo/Smac was the first protein shown to bind and antagonize IAPs.^{5,6} Recently,

HtrA2, another mammalian protein, that has an N-terminus similar to that of Grim, Reaper, HID and Diablo, has been shown to bind and antagonize IAP function.^{7,8,9,10} Unlike Grim, HID and Reaper, Diablo/Smac localizes to the intermembrane space of the mitochondria in healthy cells.^{5,6} Upon receipt of certain apoptotic stimuli, Diablo is released from the mitochondria, and in the cytosol it is able to bind and antagonize IAPs such as MIHA/XIAP.^{5,6}

Diablo is synthesized in the cytoplasm as a larger precursor protein with an N-terminal mitochondrial targeting peptide that is removed upon importation into the mitochondria.^{5,6} This processing produces an N-terminus of Diablo beginning with the residues AVP... that resembles the N-termini of Grim, Reaper and HID, which are critical for their activity.^{11,12–14} It is via these N-terminal residues that these pro-apoptotic proteins bind to the core of the baculoviral IAP repeat (BIR) domains of IAPs.^{11,12,14} Through its interaction with MIHA/XIAP, Diablo can promote apoptosis by removing inhibition of MIHA/XIAP on processed caspases 3, 7 and 9.^{11,15,16}

To determine where Diablo acts we wished to determine its tissue localization. To do this we have generated a series of monoclonal antibodies (mAbs) that can be used in tissue immunoblotting and histochemistry. Our studies show that Diablo is present in cell lines of different kinds and is detectable by immunohistochemistry in the testes, kidney and liver.

Results

Characterization of the monoclonal antibodies

Immunization of rats with mouse recombinant Diablo followed by fusion of spleen cells with the myeloma cell line SP2/0 yielded several hybridoma clones producing mAb specific for Diablo. The hybridoma clones secreting the mAb, against Diablo, were identified by flow cytometric analysis of 293T cells over-expressing HA Diablo (see Materials and Methods). Figure 1A is the staining profile of the transfected 293T cells incubated with secondary antibody alone (dotted line) or together with hybridoma supernatant which showed no immunoreactivity to Diablo (solid line). Hybridoma clones producing mAb specific for Diablo yielded a profile (Figure 1C,D) similar to the one produced when anti-HA antibody was used to detect the over-expressed HA Diablo (Figure 1B), which serves as a positive control. From an initial screen of 2000 hybridoma cultures, 20 anti-Diablo mAb generating hybridoma clones giving a profile similar to Figure 1C,D were selected for further analysis. Here we report characterization of two selected mAbs, 10G7 and 9H10. These mAbs were isotyped to be rat IgG2a, and were purified using protein-G affinity columns. They were tested for applications including Western blot

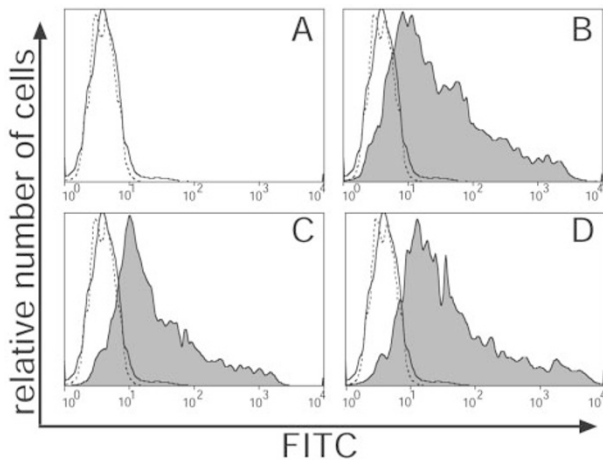


Figure 1 Screening of hybridoma clones for Diablo specific monoclonal antibodies (mAbs) by flow cytometry using HA Diablo expressed in 293T cells. 293T cells were transfected with vector encoding HA Diablo and stained with FITC-labelled secondary goat anti-rat IgG alone (dotted line in **A**) after incubation with supernatants from hybridoma clones not secreting the mAb (solid line in **A**), after incubation with anti-HA antibody (**B**) or after incubation with supernatants from hybridoma clones secreting anti-Diablo mAbs (**C** and **D**). **C–D** represent profile for the hybridoma clones producing mAbs that can detect Diablo in the cells over-expressing HA Diablo. For comparison the negative profiles shown in **A** have been super imposed in **B–D**

analysis, immunohistochemistry, immunocytochemistry and immunoprecipitation (Table 1).

The specificity of 10G7 and 9H10

Equal amounts of protein from various human and mouse cell lines was analyzed directly or after immunoprecipitation using either 10G7 or 9H10. Lysate from 293T cells over-expressing HA Diablo and probed with either of the mAb was used as size control.

Western blot and immunoprecipitation analysis of lysates from various cell lines demonstrated that 10G7 recognizes both human and mouse Diablos (Figures 2A and 3). The membrane was re-probed with anti-HA antibody to detect the over-expressed HA Diablo in lanes 6 and 7 (Figure 2B). Due to over-expression and possible overloading of the mitochondrial processing mechanisms, the transfected cells show a band for unprocessed Diablo in addition to processed HA Diablo band (23 kD), which is similar to the endogenous Diablo. The processed HA Diablo runs slower than the endogenous Diablo presumably due to addition of the 9 residue HA tag. Under similar conditions 9H10 is specific for mouse Diablo (Figure 4A). 9H10 did not detect Diablo in the three human cell lines studied here. Equal protein loading from all cell lines was demonstrated by re-probing the blots with antibody specific to Hsp70 (Figures 2C and 4B).

Expression of Diablo in mouse tissues

Endogenous mouse Diablo was detected in various tissues by Western blot and immunoprecipitation (Figures 5A and 6) using 9H10. Testes showed the highest level of expression,

Table 1 Summary of characteristics for Diablo mAbs: 10G7 and 9H10

Clone name	Isotype	Applications	Specificity
10G7	IgG2a	WB, IP, IF, FACS and Elisa	human and mouse
9H10	IgG2a	WB, IP, IF, FACS, Elisa and tissue histochemistry	mouse

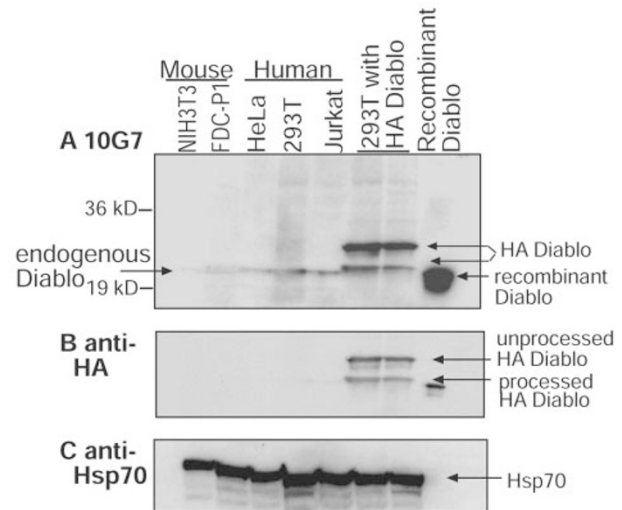


Figure 2 Detection of endogenous Diablo by Western analysis using 10G7. **(A)** Total protein (150 μ g per lane) from mouse (lanes 1–2) and human (lanes 3–5) derived cell lines was used to detect endogenous Diablo by Western blot (lanes 1–5). Lanes 6 and 7 contain whole cell lysate from 293T cells overexpressing HA Diablo. Bacterially produced recombinant protein was used in lane 8. **(B)** The membrane was re-probed with anti-HA antibodies to reveal HA Diablo in lanes 6 and 7. In addition to processed Diablo, unprocessed Diablo can also be detected in 293T cells transfected with HA Diablo construct. **(C)** Immunoblotting with antibody to Hsp 70 indicated protein loading

followed by moderate expression in kidney, liver, intestine, lung and pancreas (Figures 5A and 6). Heart also showed a low level of Diablo (although not obvious in the figure shown here). There was no visible Diablo expression in brain (Figures 5A and 6). In other tissues such as thymus, spleen and skeletal muscle Diablo was not detectable by Western analysis (data not included).

The expression pattern of Diablo in mouse tissues was also investigated by immunohistochemistry using 9H10 mAb (Figure 7). Consistent with the results obtained by immunoblotting, Diablo was most abundant in the testes. Intense Diablo immunostaining was observed in the germinal epithelium of seminiferous tubules but not in the Leydig cells of interstitium. Staining was intense in the cytoplasm of primary spermatocytes, secondary spermatocytes and spermatids (Figure 7C). In the kidney, Diablo was most abundant in the ductal epithelia of the cortex but no immunoreactivity was detected in the glomeruli (Figure 7A). In the liver, the expression of Diablo was restricted to hepatocytes (Figure 7B).

Diablo localizes to the mitochondria

HeLa cells were transiently transfected with the full-length mouse Diablo pEF construct (see Materials and Methods) and immunofluorescence staining was performed using 9H10 followed by detection with FITC-labelled secondary antibody. The mitochondria and nuclei were co-stained using Mito Tracer Red and DAPI respectively. Analysis was performed using confocal microscopy. While the mitochondria and nuclei of all cells were stained, 9H10 only detected mouse Diablo in the cells transfected with the mouse Diablo construct. Under the conditions used here, 9H10 does not react with endogenous human Diablo in HeLa cells (Figure 8A,B). Furthermore, in healthy cells (Figure 8A) most of the Diablo co-localized within the mitochondria whereas after exposure to UV, Diablo was present throughout the cell (Figure 8B). Using 10G7, distribution of endogenous human Diablo in

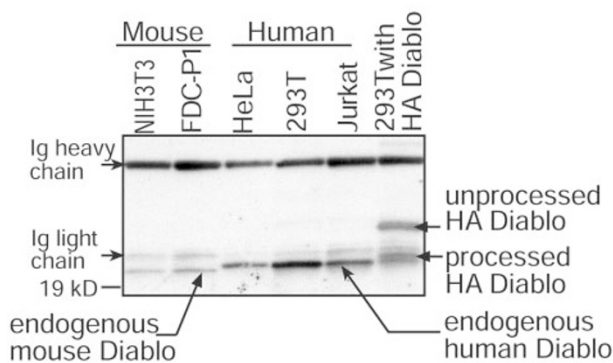


Figure 3 Immunoprecipitation of endogenous Diablo from cell lines using 10G7. Equal amounts of protein from mouse (lanes 1–2) and human cell lines (lanes 3–5) were immunoprecipitated using 10G7, subjected to electrophoresis, blotted and detected with polyclonal rabbit antiserum to Smac. Lane 6 contains protein from 293T cells over-expressing HA Diablo that was immunoprecipitated with 10G7

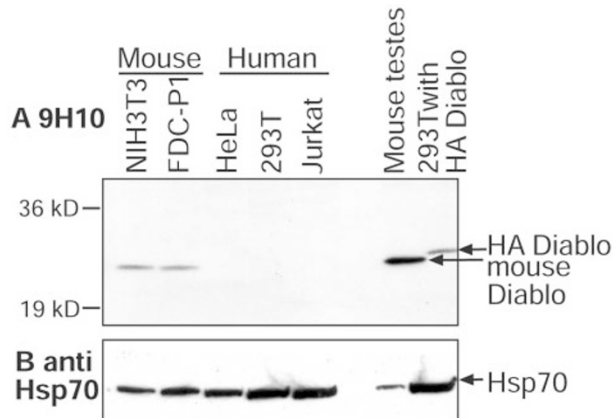


Figure 4 Detection of endogenous Diablo from cell lines by Western blotting using 9H10. (A) 150 μ g of total protein from mouse (lanes 1–2) and human cell lines (lanes 3–5) were probed with 9H10. Whole cell lysates from mouse testes (lane 7) and 293T cells over-expressing HA Diablo (lane 8) were used as positive controls. (B) The membrane was re-probed with anti Hsp 70 to demonstrate protein loading

HeLa cells showed a punctate expression consistent with mitochondrial localization (data not shown).

Discussion

Full-length Diablo (27 kD) undergoes N-terminal processing to yield a smaller (21 kD) protein.⁶ As seen in Figures 2A and 3, endogenous mouse Diablo seems to run slightly faster than endogenous human Diablo even though Diablo in the two species has a similar predicted molecular weight and 89% identity.

The amounts of Diablo protein in mouse tissues correlated well with the mRNA levels as previously described^{5,6} except in the heart, where the amount of Diablo protein seems much lower than that expected from the abundance of mRNA. Testes showed the highest levels

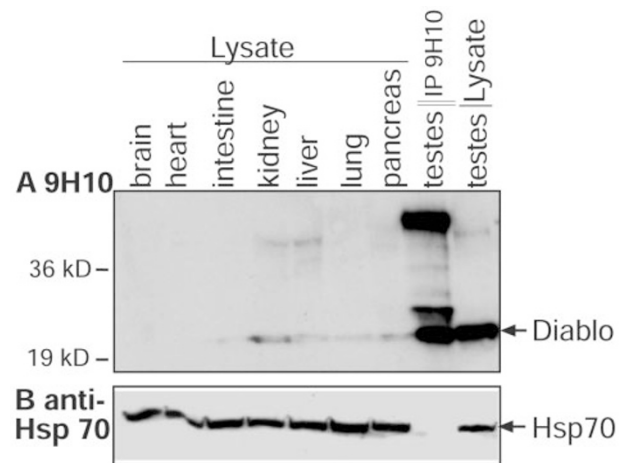


Figure 5 Detection of endogenous Diablo in mouse tissues using 9H10. (A) 300 μ g of total protein from various tissues was analyzed by direct Western blot using 9H10 (lanes 1–7 and 9). For size comparison, lane 8 contains Diablo immunoprecipitated from mouse testes using 9H10 followed by Western blot with the same antibody. In addition to detection of endogenous Diablo, bands for IgG heavy and light chain can also be seen in lane 8. (B) An identical gel was run for probing with anti Hsp 70 to indicate protein loading

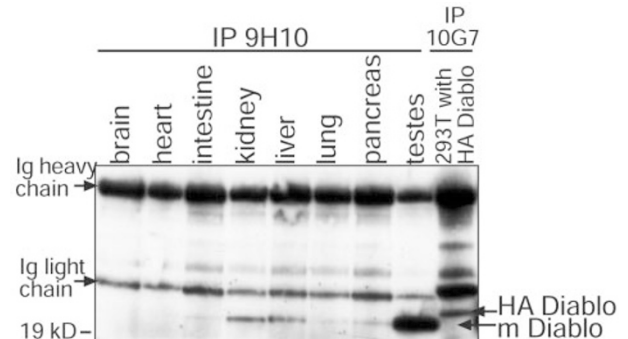


Figure 6 Immunoprecipitation of Diablo from mouse tissues using 9H10. Mouse tissue proteins (500 μ g) were immunoprecipitated using 9H10 (lanes 1–8) and detected with 9H10. As a positive control, lane 9 contains Diablo immunoprecipitated with 10G7 from 293T cells transfected with HA Diablo construct prior to detection by 9H10

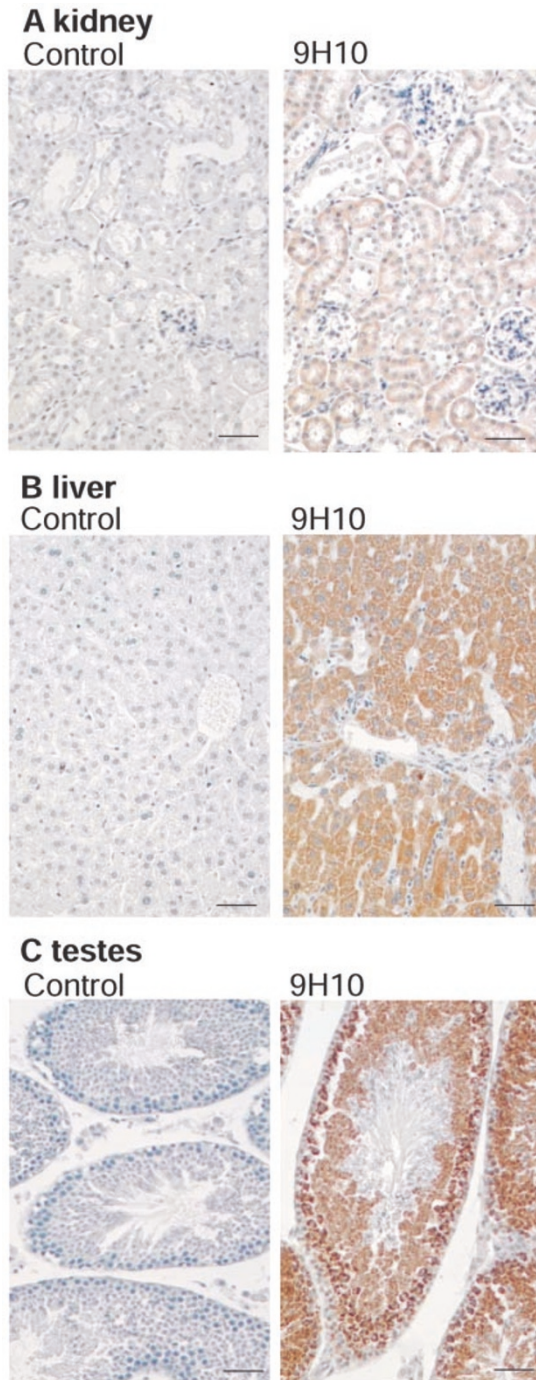


Figure 7 Tissue expression of mouse Diablo as revealed by immunohistochemistry. Using 9H10 endogenous mouse Diablo was detectable in the kidney (A), liver (B) and testes (C). Biotinylated goat anti-rat IgG was used as the secondary antibody followed by detection with DAB reagent. The bar represents 200 μm

of Diablo protein. It is possible that high expression of pro-apoptotic molecules, such as Diablo, may be responsible for conferring the high sensitivity of testicular germ cells to radiation-induced apoptosis^{17,18} however Diablo was not abundant in the thymus, where cell turn over is also high and cells are similarly sensitive to radiation.

The pro-apoptotic activity of Diablo is mainly controlled by its localization. Diablo is produced as a precursor protein and targeted to the mitochondria by an N-terminal targeting peptide, which is removed by proteolysis to localize Diablo to the inter-membrane space.^{5,6} The processed form is present in the mitochondria of healthy cells, as is cytochrome c, which when released can activate Apaf-1 in the cytosol, leading to activation of the apoptosome and caspases 9 and 3.^{5,6,19,20} Processing of Diablo produces an N-terminus beginning with the residues AVP that resembles the N-termini of *Drosophila* pro-apoptotic proteins Grim, Reaper and HID that are critical for their activity.^{11–14} As shown in Figure 8, over-expressed mDiablo detected by mouse specific 9H10 localizes to the mitochondria. Endogenous human Diablo in HeLa cells, detected by 10G7 had the same pattern of expression as seen in the transfected cells (data not shown). Following an apoptotic stimulus such as UV radiation, Diablo is released from the mitochondria into the cytoplasm.^{5,6} In the cytoplasm Diablo can interact with IAPs^{5,6} thereby preventing them from inhibiting activated caspases.^{21–23}

The finding that Diablo is expressed differentially among various tissue types, and the lack of correlation between levels of Diablo mRNA and protein in the heart, raises the possibility that Diablo may be under transcription and post-translational control, in addition to being controlled by its localization within the mitochondria. Indeed, a recent publication suggests that the Diablo gene may be induced by the cytokine IFN γ .²⁴

Materials and Methods

Expression constructs

Full-length mouse Diablo cDNA fused to a C-terminal HA (YPYDVPDYA) epitope tag (HA Diablo) subcloned into mammalian expression vector pcDNA3 as described previously⁶ was used for expression in the 293T cell line. A pEF BOS expression construct encoding untagged Diablo also described previously⁶ was used for expression in HeLa cells. To prepare recombinant protein, Diablo was expressed as a glutathione-S-transferase (GST) fusion protein. N-terminal deleted fragment (1–54) of mouse Diablo cDNA, encoding the processed form (without the mitochondrial targeting sequence), was sub-cloned into vector pGEX-6p-3 (Pharmacia) for production of GST-Diablo in *E. coli* BL21.

Protein expression and purification

GST-Diablo was expressed and purified according to the previously described procedure.^{25,26} Briefly, the recombinant GST-Diablo protein was expressed in *E. coli* BL21 (DE3) following IPTG induction. The cells were lysed and protein purified by affinity chromatography using glutathione sepharose (Pharmacia). While the sample was bound to the resin, the GST-tag was removed by overnight digestion with PreScission protease (Amersham Pharmacia). Soluble Diablo was recovered from the resin and further purified by size exclusion chromatography using a Superdex 200 column. Following the purification Diablo contained five additional residues (GPLGS) attached to its N-terminus. The purified recombinant Diablo was used to immunize the rats for production of monoclonal antibodies.

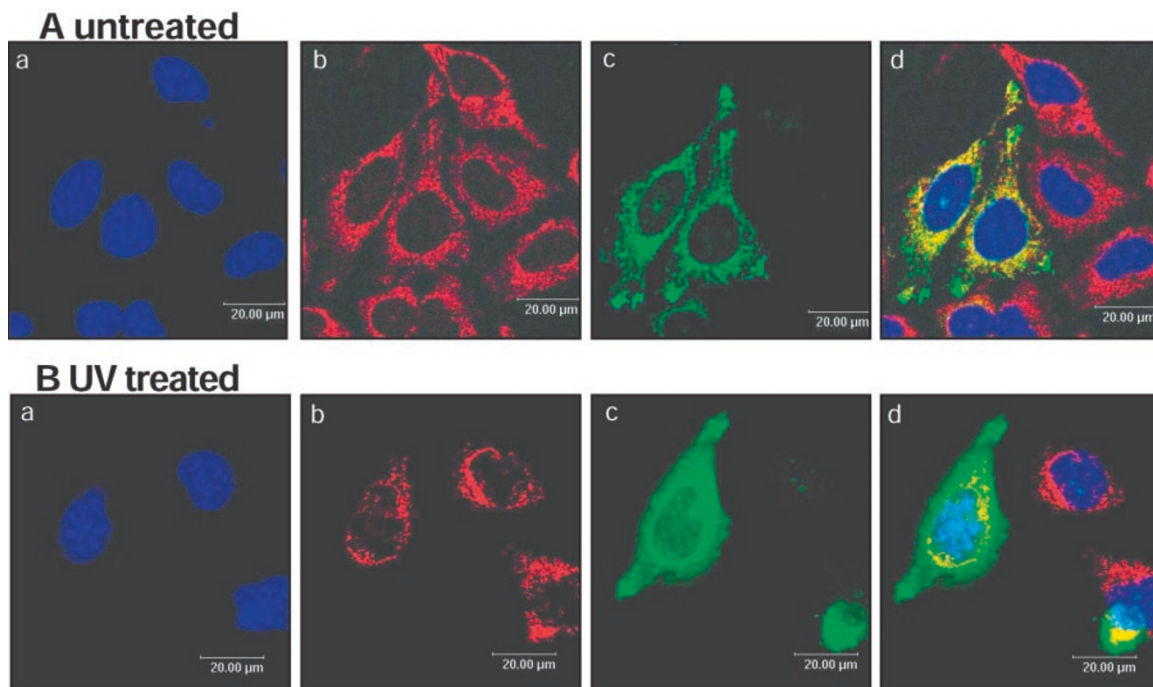


Figure 8 Localization of Diablo in HeLa cells using confocal microscopy. HeLa cells were transfected with a vector containing mouse Diablo and stained with 9H10, followed by a FITC-coupled secondary (c). Mitochondria and nuclei were co-stained using Mito Tracer Red (b) and DAPI (a). **(A)** In healthy cells Diablo is mainly localized to mitochondria (c,d). **(B)** In UV treated cells Diablo leaks out from the mitochondria (c,d)

Immunization

Wistar rats were initially immunized by subcutaneous (s.c.) injection with 100 µg of purified recombinant Diablo mixed with complete Freund's adjuvant (Difco). Two subsequent boosts of the immunogen, resuspended in complete Freund's adjuvant (Difco), were injected s.c., 3 and 6 weeks after the initial injection. A final boost with recombinant Diablo dissolved in PBS was given intravenously (i.v.) and intraperitoneally (i.p.) after 4 weeks. Three days later, hybridomas were generated by fusing spleen cells from immunized rats with the SP2/0 myeloma cell line as previously described.²⁷

Hybridoma screening for antibodies to Diablo

Hybridomas producing monoclonal antibodies to Diablo were identified and their isotype determined using a previously described screening strategy.²⁷ Briefly, 293T cells were transiently transfected with vectors encoding HA-tagged Diablo. 5×10^5 transfected cells were fixed in 1% paraformaldehyde and plated into each well of 96-well tissue culture plate (Becton Dickinson, CA, USA). The cells were washed, permeabilized with 0.3% saponin (Sigma) and incubated with 100 µl of hybridoma supernatants for 1 h on ice. Bound antibodies were revealed with fluorescein-isothiocyanate (FITC)-conjugated goat anti-rat IgG (H+L) secondary antibodies (Southern Biotechnology) and detected by FACScanTM (Becton Dickinson). Antibody to the HA epitope tag (Boehringer Mannheim) was used as positive control to detect efficiency of transfection of HA Diablo construct and compare the profile of the antibody producing clones.

Hybridomas producing antibodies to Diablo were cloned twice and adapted for growth in medium containing low serum. For production of large amounts of antibodies, hybridomas were cultured for several weeks in the miniPERM classic 12.5 kD bioreactors (VIVA SCIENCE). Antibodies were purified on a protein-G sepharose column (Pharmacia).

Tissue culture and transfections

The human embryonic carcinoma cell line 293T and mouse cell lines NIH3T3 and FDC-P1 were routinely grown in DME media supplemented with 10% FCS. FDC-P1 cells were also supplemented with IL-3. Human cell lines HeLa and Jurkat were grown in RPMI media supplemented with 10% FCS. These cell lines were used to detect endogenous Diablo by immunoprecipitation and Western analysis.

293T and HeLa cells were transfected with HA Diablo or untagged Diablo construct using EffecteneTM transfection reagent (Qiagen) following the manufacturer's instructions. Forty-eight hours after transfection, the cells were used for screening the hybridoma clones and/or examining the expression of transfected Diablo constructs by immunoprecipitation, immunofluorescence and Western analysis.

Western blotting and immunoprecipitation

Various mouse organs/tissues were excised, rinsed in PBS and immediately frozen in isopentane on dry ice before homogenizing in lysis buffer at 4°C according to the method described previously.²⁸ Cell lysates were prepared from various cell lines or mouse tissues using cold lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 10% Triton X-100, 10% glycerol, 2 mM EDTA and complete protease inhibitor cocktail obtained from Roche, Mannheim, Germany). The concentration of protein was determined using Bio-Rad protein assay reagent (Bio-Rad laboratories, NSW, Australia). These lysates were then used for Western blots or for immunoprecipitation. One hundred and fifty micrograms of total protein from various cell lines was used to detect endogenous Diablo by Western blot. A minimum of 300 µg of total mouse tissue protein per lane was required to detect endogenous Diablo by Western blot. Protein from the cell lysates (a minimum of 500 µg from tissue total protein) was immunoprecipitated with anti-Diablo 10G7 or 9H10 mAbs plus protein G-Sepharose for 1 h. After

extensive washing the immunoprecipitated material was eluted by boiling in sodium dodecyl sulphate-polyacrylamide gel loading buffer. Protein samples were size-fractionated on polyacrylamide gels (Gradipore), and transferred to HybondTM-C extra membranes (Amersham Life Science) by electroblotting. Non-specific binding of antibodies to membranes was blocked by incubation in 10% skimmed milk or 10% horse serum made in PBS containing 0.05% Tween-20. The membranes were then probed with 10G7 or 9H10 followed by HRP labelled goat anti-rat IgG (Amersham Pharmacia or Southern Biotechnology). Bound antibodies were visualized by enhanced chemiluminescence (ECL) (Amersham Pharmacia). Transfected HA Diablo was used as a size control for most of the experiments. To control for the concentration and integrity of proteins in the tissue lysates, blots were reprobbed with mouse anti-Hsp70 mAb (a gift from Dr. David Huang, The Walter and Eliza Hall Institute, Melbourne, Australia). The rabbit antiserum to Diablo/Smac was kindly provided to us by Dr. Xiaodong Wang.⁵

Tissue histochemistry

Tissue histochemistry procedures were based on online protocols (www.protocol-online.net/immuno/immunohisto/immunohistochemistry.htm). Freshly cut mouse tissues were fixed in Histochoice (Amresco, Solon, OH) for 1–3 h and processed for paraffin embedding followed by section cutting. Before staining the tissue sections were dewaxed and gradually rehydrated. Tissue sections were treated with 0.3% hydrogen peroxide in PBS to block the endogenous peroxidase activity. The cells were then permeabilized by incubation in 0.2% Triton X-100 and non-specific staining was prevented by incubation with 2.5% horse serum for 1 h at room temperature. Sections were incubated in a humidified chamber with 9H10 for 1 h at room temperature. After washing in PBS containing 0.2% Triton X-100, tissues were incubated with HRP conjugated goat anti-rat IgG (Amersham Pharmacia). To enhance the signal, the tissues were incubated with biotinylated goat anti-rat IgG (Southern Biotechnology). This was followed by incubating with ABC Vectastain elite reagent (Vector Laboratories, CA, USA) and finally the sections were stained with DAB reagent (Vector Laboratories, CA, USA) according to the manufacturer's instructions. Sections were counter-stained with hematoxylin, and dehydrated in graded concentrations of alcohol and histolene before mounting in DPX (BDH, Poole, UK).

Immunofluorescence

HeLa cells were grown to about 30% confluency on cover slips followed by transient transfection with vectors encoding mouse Diablo. After 40 h cells were either exposed to UV (25 J/M²) to induce UV radiation-induced apoptosis or left unexposed. Seven hours later all cells were incubated with Mito Tracker red (Molecular Probes) for 15 min at 37°C to stain the mitochondria before fixing in Histochoice (Amresco, Solon, OH, USA) for 10 min. Expression of Diablo was detected using 9H10 for 1 h followed by FITC labelled goat anti-rat (Southern Biotechnology). Nuclei were stained by adding DAPI, for 30 min, together with the secondary antibody. Microscopy was performed on a confocal microscope (Leica Microsystems, Germany) visualizing mitochondria (as red), Diablo (as green) and nucleus (as blue).

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