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Outer mitochondrial membrane localization of apoptosis-inducing factor: mechanistic implications for release

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ABSTRACT

Poly(ADP-ribose) polymerase-1-dependent cell death (known as parthanatos) plays a pivotal role in many clinically important events including ischaemia/reperfusion injury and glutamate excitotoxicity. A recent study by us has shown that uncleaved AIF (apoptosis-inducing factor), but not calpain-hydrolysed truncated-AIF, was rapidly released from the mitochondria during parthanatos, implicating a second pool of AIF that might be present in brain mitochondria contributing to the rapid release. In the present study, a novel AIF pool is revealed in brain mitochondria by multiple biochemical analyses. Approx. 30% of AIF loosely associates with the outer mitochondrial membrane on the cytosolic side, in addition to its main localization in the mitochondrial intermembrane space attached to the inner membrane. Immunogold electron microscopic analysis of mouse brain further supports AIF association with the outer, as well as the inner, mitochondrial membrane *in vivo*. In line with these observations, approx. 20% of uncleaved AIF rapidly translocates to the nucleus and functionally causes neuronal death upon NMDA (*N*-methyl-D-aspartate) treatment. In the present study we show for the first time a second pool of AIF in brain mitochondria and demonstrate that this pool does not require cleavage and that it contributes to the rapid release of AIF. Moreover, these results suggest that this outer mitochondrial pool of AIF is sufficient to cause cell death during parthanatos. Interfering with the release of this outer mitochondrial pool of AIF during cell injury paradigms that use parthanatos hold particular promise for novel therapies to treat neurological disorders.

Key words: apoptosis-inducing factor (AIF), immunogold electron microscopy, mitochondrion, parthanatos, poly(ADP-ribose) polymerase-1.

INTRODUCTION

Mitochondria play a critical role in cell death via regulating the release of pro-apoptotic proteins including cyt c (cytochrome *c*) and AIF (apoptosis-inducing factor) (Martinou and Green, 2001). AIF was initially thought to be located in the intermembrane space of mitochondria in a 57 kDa soluble form (Susin et al., 1999). However, Otera et al. (2005) showed that mature AIF, which contains a hydrophobic transmembrane segment, is a 62 kDa type-I inner membrane protein with the N-terminus exposed to the matrix and the C-terminal portion exposed to the intermembrane space. To be released from the mitochondria, AIF would then need to be processed into a 57 kDa soluble form by removing the hydrophobic transmembrane segment.

AIF is a critical mediator for PARP-1 [poly(ADP-ribose) polymerase-1]-initiated cell death (known as parthanatos). PARP-1 is a nuclear enzyme which acts as a DNA damage sensor (Lautier et al., 1993; de Murcia et al., 1994; Shall and de Murcia, 2000). PARP-1 overactivation mediates cell death under different pathological conditions, including ischaemia/reperfusion injury (Eliasson et al., 1997; Pieper et al., 2000),

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Abbreviations: AIF, apoptosis-inducing factor; ANT, adenine nucleotide translocator; cyt c, cytochrome c; Hq, Harlequin; MnSOD, manganese superoxide dismutase; NMDA, *N*-methyl-D-aspartate; PARP-1, poly(ADP-ribose) polymerase-1; ROI, region of interest; Smac, second mitochondrial-derived activator of caspase; Tim23, translocase of inner membrane 23; Tom20, translocase of outer membrane 20; VDAC, voltage-dependent anion channel; WT, wild-type.

glutamate excitotoxicity (Mandir et al., 2000; Yu et al., 2002; Wang et al., 2004), oxidative stress, inflammation (Szabo and Dawson, 1998) and in models of Parkinson's disease (Cosi and Marien, 1999; Mandir et al. 1999). In parthanatos, the key event is AIF release from the mitochondria and translocation to the nucleus, followed by chromatin condensation, large-scale DNA fragmentation and subsequent cell death (Wang et al. 2009a). Calpain, a calcium-dependent intracellular cysteine protease, has been suggested to play an important role in mitochondrial AIF release under certain cell-death pathways via hydrolysing mature AIF to 57 kDa truncated AIF (Polster et al., 2005; Cao et al. 2007; Moubarak et al. 2007). However, a recent study by us has shown that it is the 62 kDa uncleaved mature AIF that is released from the mitochondria to the nucleus, and calpain was not required for mitochondrial AIF release in parthanatos (Wang et al. 2009b). Inhibition of calpain with calpastatin or genetic knockout of the regulatory subunit of calpain failed to prevent NMDA (*N*-methyl-D-aspartate)- or MNNG (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine)-induced AIF nuclear translocation and subsequent cell death. In line with our observation, another group has also shown that mitochondrial μ-calpain is not involved in the processing of AIF (Joshi et al. 2009). Additionally, AIF release is detected before cyt c in parthanatos in spite of its larger molecular mass, 57–67 kDa compared with 14 kDa respectively (Yu et al., 2002, 2003; Wang et al., 2004). Taken together, these observations suggest that a second pool of AIF might exist in brain mitochondria which contributes to the rapid release as the uncleaved form during parthanatos.

In the present paper we report that endogenous AIF has two major submitochondrial localizations in brain. One is at the outer mitochondrial membrane on the cytosolic side, and the other localization is in the intermembrane space, attached to the inner membrane. There is little detectable free, soluble AIF in the intermembrane space. The present finding of two pools of mitochondrial AIF expand the current concept of mitochondrial AIF, and may provide a better understanding of AIF release under different cell-death paradigms.

MATERIALS AND METHODS

Preparation and treatment of mitochondria from brain

All animal experiments were approved by the Animal Care and Use Committee at the John Hopkins University School of Medicine. Non-synaptosomal mitochondria were prepared from adult mouse or rat brains (6–8 weeks of age) using the Percoll gradient method as described previously (Kristian and Fiskum, 2004). Respiration of mitochondrial preparations were evaluated with a Clark-type oxygen electrode (Polster et al., 2001). The final mitochondria stock was prepared in isotonic buffer [300 mM sucrose and 10 mM Hepes (pH 7.4), without EGTA]. Rat brain was used for submitochondrial fractionation experiments using a French press (Thermo). For the alkaline treatment, mitochondrial pellets were resuspended in 0.1 M Na_2CO_3 (pH 11.5) and incubated on ice for 20 min. The soluble supernatant and insoluble membrane fractions were separated by ultracentrifugation [37 000 rev./min (TLA-120.2 rotor) for 30 min]. For salting-out experiments, the mitochondrial fraction (3 mg/ml) was divided into several aliquots, centrifuged at 12 000 *g* for 5 min, and the pellets were resuspended in the salting-out buffer [250 mM sucrose, 25 mM KCl, 5 mM $MgCl_2$, 0.1 mM PMSF, 10 mM Tris/HCl (pH 7.4) and protease inhibitors (Roche) containing different concentrations of NaCl]. For the proteinase K treatment, the mitochondria were prepared without protease inhibitor and were incubated with 1 μg of proteinase K at 37°C. The antibodies against AIF and MnSOD (manganese superoxide dismutase) have been described previously (Yu et al., 2002; Wang et al., 2004). Commercially obtained antibodies were: rabbit anti-AIF monoclonal antibody (Epitomics; Epi), mouse anti-AIF monoclonal antibody (E-1), anti-ANT (adenine nucleotide translocator), anti-Tom20 (translocase of outer membrane 20) and anti-Tim23 (translocase of inner membrane 23) (all from Santa Cruz Biotechnology), anti-VDAC (voltage-dependent anion channel) (Calbiochem), anti-Smac (second mitochondrial-derived activator of caspase; Chemicon) and anti-cyt c (BD Pharmingen).

Submitochondrial fractionation

Mitochondria prepared from rat brains were resuspended in 3.5 ml of isotonic buffer, transferred to the French press and a pressure of 16000 psi (1 psi=6.9 kPa) was applied. Mitochondria were homogenized with a flow valve rate of 15 drops/min. Lysate was centrifuged at 12 000 ***g*** for 10 min and the pellet was saved as the mitoplast fraction. The supernatant was subjected to ultracentrifugation at 59 000 rev./min (TLA-120.2 rotor) for 1 h and further fractionated into the intermembrane space and outer membrane fractions respectively. Equal amounts of protein from each fraction were loaded for immunoblot analysis.

Electron microscopy

Mice were perfused through the heart with 4% formaldehyde and 0.1% glutaraldehyde in phosphate buffer. Mouse neocortices were cut into 0.5–1 mm slices, cryoprotected, quick-frozen in liquid propane (−170°C), and subjected to freeze substitution. Specimens were embedded in methacrylate resin (Lowicryl HM20) and polymerized by UV light below 0°C. Ultrathin sections were incubated with rabbit monoclonal antibodies against AIF (10 μg/ml; Epi) followed by goat anti-rabbit antibody coupled to 15 nm Colloidal Gold. The sections were examined in a Philips CM 10 electron microscope at 60 kV.

Cell culture, subcellular fractionation preparation and cytotoxicity

Primary neuronal cultures from cortex were prepared as described previously (Dawson et al., 1993). At 2 h after NMDA (Sigma) treatment (500 μM for 5 min), cells were harvested. Nuclear subcellular fractions and post-nuclear subcellular fractions, which includes mitochondria and cytosol, were prepared (Wang et al., 2004). PARP-1-dependent cell death was induced by 500 μM NMDA for 5 min. Viability was assessed 24 h after treatment with Hoechst 33342 (7 μM; for total nuclei) and propidium iodide (2 μM; for dead cell nuclei) double staining.

Quantification and statistical analysis

Immunogold labelling was quantified with analySIS (Soft Imaging Systems) from digital images of two sections from two mouse neocortices (50 mitochondria from each section) acquired in a blinded manner. Mitochondrial labelling was recorded as the number of gold particles per unit area and determined by an extension of analySIS. ROIs (regions of interest) were drawn interactively, and ROI results determined semi-automatically and transferred to SPSS version 13. Significance was determined using a Student's unpaired *t* test; $P<0.05$ was considered statistically significant. Data are the means $\pm$ S.E.M.

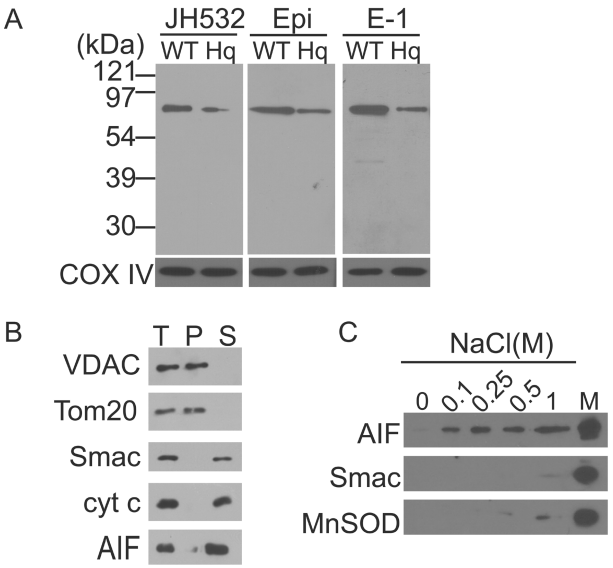

Figure 1 Biochemical determination of AIF localization in brain mitochondria

(**A**) Immunoblot of mitochondrial fractions (5 μg each) from WT and Hq mice brains probed with anti-AIF antibodies: JH532, Epi and E-1. The molecular mass in kDa is indicated on the left-hand side. COX IV, cyt c oxidase subunit IV. (**B**) The insoluble pellet (P) and soluble supernatant (S) of an alkaline extraction of brain mitochondria extraction probed for AIF, the membrane proteins VDAC and Tom20, and the soluble proteins Smac and cyt c. T, total mitochondria. (**C**) Salting-out experiment. AIF is washed out with the increasing concentrations of NaCl, whereas MnSOD and Smac remain in the pellet. M (in lane 6), total mitochondria.

RESULTS

Sub-mitochondrial localization of AIF

AIF has been reported in the intermembrane space of mitochondria (Susin et al., 1999). Previously we have observed that AIF release occurs before that of cyt c and caspase activation after DNA damage and NMDA excitotoxicity (Yu et al., 2002; Wang et al., 2004). Since AIF is a much larger protein than cyt c, the relative kinetics of AIF translocation to the nucleus compared with cyt c release from mitochondria was puzzling. To address this issue, the localization of AIF was examined at submitochondrial levels in rodent brain. Three different AIF antibodies were used JH532 (Wang et al., 2004), an rabbit anti-AIF monoclonal antibody (Epi) and a mouse anti-AIF monoclonal antibody (E-1). To confirm the specificity of these antibodies, mitochondria were prepared from WT (wild-type) or Hq (Harlequin) mice, which have substantially reduced levels of AIF due to a proviral insertion (Klein et al., 2002). All three antibodies recognized only one band of the same molecular mass (62 kDa) on immunoblot, with decreased intensity in Hq samples (Figure 1A). A Percoll gradient was used to obtain intact mitochondria for *in vitro* studies. For the alkaline treatment, purified mitochondria were incubated with 0.1 M Na_2CO_3 (pH 11.5). In this experiment, VDAC and Tom20, which are integral mitochondrial membrane proteins, served as markers and were retained in the membrane fraction. In contrast, AIF was found in the soluble supernatant along with the soluble mitochondrial proteins, Smac and cyt c (Figure 1B), suggesting that AIF is not an integral membrane protein. For salting-out experiments, mitochondria were incubated in buffers containing different concentrations of NaCl. After pelleting mitochondria by centrifugation, the supernatants were probed with anti-AIF antibodies (Figure 1C). Increasing concentrations of NaCl led to dissociation of AIF from mitochondria (Figure 1C). In contrast, Smac and MnSOD, which are mitochondrial intermembrane space and matrix proteins respectively, were not detected in the supernatant over the concentration range of NaCl, with barely detected levels at high-salt concentrations. These results suggest that high-salt treatment does not alter mitochondrial integrity and that there is a pool of AIF that is loosely associated with the outer membrane of mitochondria on the cytosolic side.

Purified mitochondria were treated with proteinase K to degrade exposed proteins (Figure 2A). Much of Tom20 faces the cytosol, anchored by an N-terminal transmembrane segment. Tom20 is highly sensitive to proteinase K, whereas the intermembrane space protein, Smac, is protected by the outer membrane and is insensitive to proteinase K (Figure 2A). Notably, AIF is moderately sensitive to proteinase K with $72.6 \pm 4.5\%$ (mean for five independent experiments) of AIF remaining following treatment, suggesting that a small

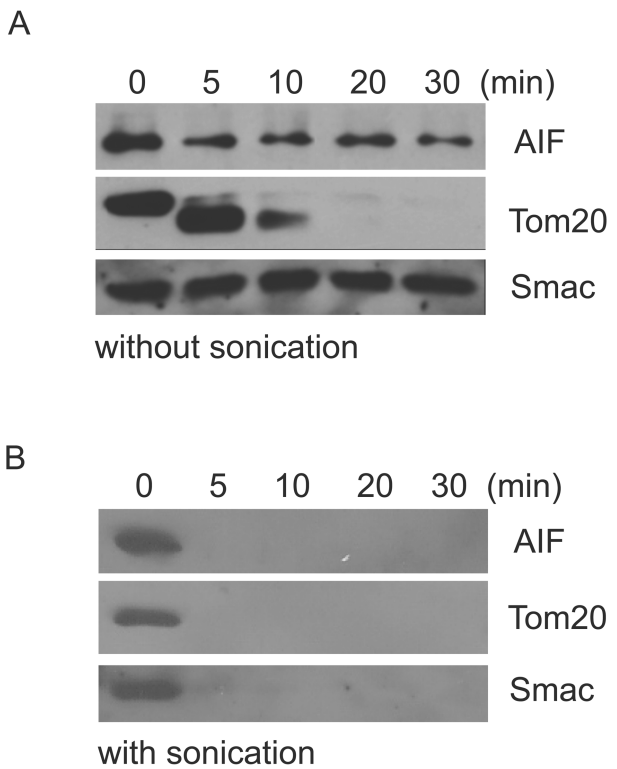

Figure 2 AIF is localized, in part, to the outer mitochondrial membrane
(**A**) Brain mitochondria (400 μg) were treated with proteinase K (1 μg) and immunoblotted to detect AIF, Tom20 and Smac. Tom20 and Smac are resistant to protease K. (**B**) Disruption of mitochondrial structure by brief sonication renders all proteins sensitive to proteinase K. Experiments were repeated three times with similar results.

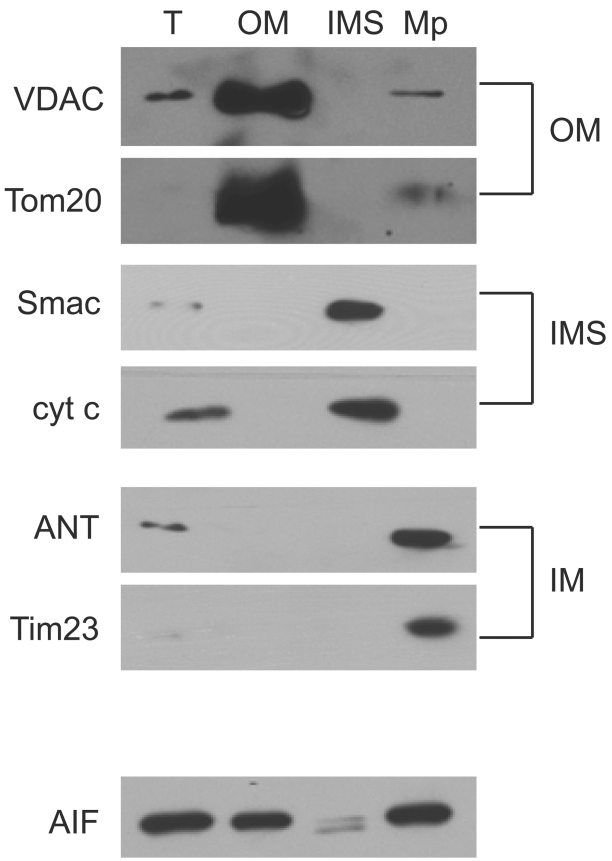

Figure 3 Submitochondrial fractionation
Mitochondria (T) isolated from the rat brain were further separated into the outer membrane fraction (OM), intermembrane space fraction (IMS) and mitopast (Mp; inner membrane and matrix) using a French press. For immunoblot analysis, 5 μg of protein from each fraction was probed for AIF, VDAC and Tom20 (outer membrane), Smac and cyt c (intermembrane space), and ANT and Tim20 (inner membrane) respectively. Experiments were repeated three times with similar results.

pool of AIF resides at the outer mitochondrial membrane (Figure 2A). All proteins are degraded by proteinase K after disruption of mitochondria by sonication (Figure 2B), indicating that the difference in their sensitivity to proteinase K is not due to their ability to be proteolytically degraded, but due to their differential localization. Taken together, these results suggest that a pool of AIF is associated with the cytosolic surface of the outer membrane in brain mitochondria.

To further examine the localization of AIF, submitochondrial fractionations were obtained using a French press. VDAC and ANT are the core components of the mitochondrial permeability transition pore complex at the outer and inner membranes respectively (Lemasters et al., 1998; Crompton, 1999). Tom20 and Tim23 are part of the mitochondrial protein transport machinery at the outer and inner membranes respectively (Mokranjac and Neupert, 2005). Smac and cyt c are pro-apoptotic proteins located within the intermembrane space. The French press generates a physical shearing force to skin mitochondria of the outer membrane without disrupting membrane integrity which occurs during osmotic swelling or detergent treatments (Hoppel et al., 1998). Under these conditions, AIF is found in both outer membrane and inner membrane fractions (Figure 3). However, the intermembrane space is the least likely localization for AIF. This suggests that, in accordance with other reports (Arnoult et al., 2002), AIF is associated with the inner membrane rather than soluble in the intermembrane space. To judge the efficacy of separation, the fractions were subjected to immunoblot analyses with antibodies against the marker proteins. The marker proteins were highly enriched at the expected fractions, demonstrating efficient and clean separation of outer membrane, intermembrane space and inner membrane fractions (Figure 3).

Electron microscopic localization of AIF

To confirm the results of the biochemical studies, we used post-embedding electron microscopy with a rabbit anti-AIF monoclonal antibody. Analysis of ultrathin sections of WT and Hq mice brains give similar results as the biochemical approaches, revealing reproducible association of gold particles with the outer, as well as the inner, mitochondrial membrane in WT mice (Figure 4). Counting particles in the corridor between 0 and 30 μm of the outer membrane

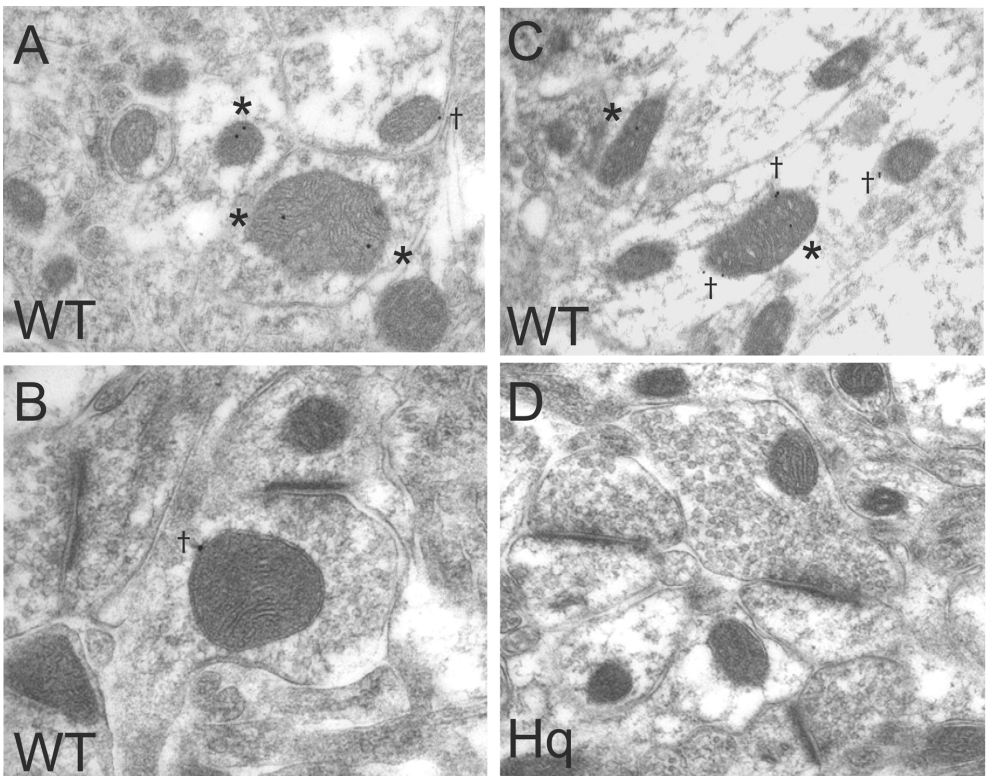

Figure 4 **AIF immunogold labelling in mouse neocortex**
Ultrathin Lowicryl sections from WT (**A–C**) or Hq (**D**) mouse neocortex were subjected to immunogold cytochemistry. Most of the gold particles are detected over inner membranes of mitochondria (indicated by *), but some mitochondria show gold particles associated with the outer membrane (indicated by †) in WT mice. Particles are rarely found in mitochondria of Hq mice.

indicates that at least 12% of the antigen pool is associated with the outer membrane. The Hq mouse brain shows very few immunogold particles (Figure 4). The number of gold particles per mitochondria was quantified for WT (4.2 ± 0.97 gold particles/mitochondria) and for Hq (0.08 ± 0.07 gold particles/mitochondria) mice ($n=50$ for each) providing evidence for specificity and selectivity of the antibody used in these experiments.

Excitotoxic releasable pool of AIF in cortical neurons

To explore whether the outer membrane pool of AIF is released during glutamate excitotoxicity, cultured primary cortical neurons were exposed to a neurotoxic concentration of 500 μM NMDA. Immunoblot analysis of nuclear fractions and postnuclear fractions, which includes cytoplasm and mitochondrial proteins, revealed translocation of approx. 20% of the total AIF to the nucleus as the uncleaved form (Figures 5A and 5B), which is consistent with the estimation of the outer membrane AIF pool by the various protein biochemical approaches and electron microscopy. Moreover, NMDA treatment at 500 μM for 5 min was sufficient to cause cell death (Figure 5C), which could be prevented by the PARP inhibitor DPQ (Figure 5C) or genetic knockout of PARP-1 via blocking AIF release from the mitochondria (Yu et al., 2002; Wang et al., 2009b).

DISCUSSION

In parthanatos, AIF is quickly released from mitochondria. Our data from the alkaline extraction assay, high-salt concentration treatment, protease treatment, submitochondrial fractionation and electron microscopy demonstrate that a fraction of AIF is attached to the outer membrane surface of brain mitochondria. This localization of AIF may allow the rapid release of AIF prior to the later release of integral mitochondrial proteins in parthanatos. The immunogold particles labelling AIF are exclusively located to the mitochondria. Although the predominant immunogold signal is at the inner membrane, particles are consistently observed at the outer membrane. Gold particles associated with the outer aspect of the outer membrane are separated by more than 25 nm from the inner mitochondrial membrane. These particles cannot be attributed to epitopes in the inner membrane, as ~25 nm corresponds to the maximum distance between a 15 nm gold particle and the respective epitope (Matsubara et al., 1996). Because of high protein content, specificity of immunogold labelling of mitochondria should always be assessed with extreme care. In the present study, the Hq mice with 80% down-regulation of AIF expression (Klein et al., 2002) are an invaluable tool to verify the

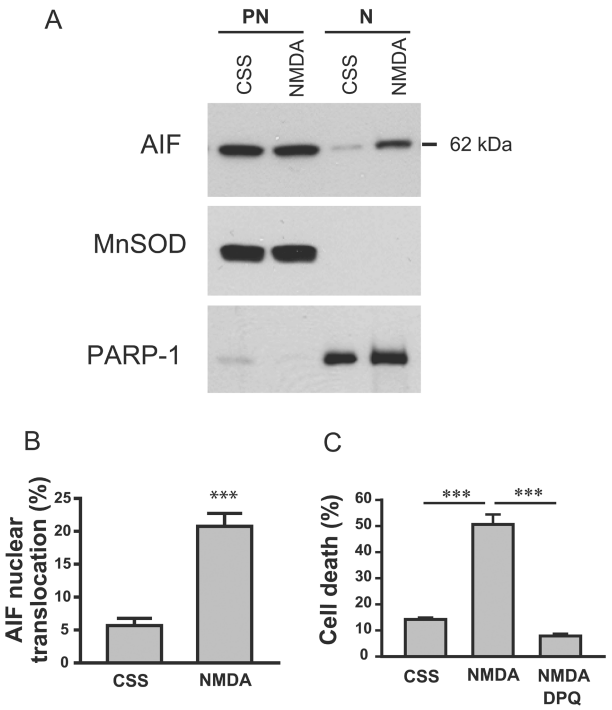

Figure 5 AIF nuclear translocation in cortical neurons

(**A**) Representative immunoblots of NMDA (500 μM for 5 min) caused AIF nuclear translocation in cortical neurons. PN, postnuclear fraction; N, nuclear fraction; CSS, controlled salt solution. MnSOD and PARP-1 were used as markers of postnuclear and nuclear fractions respectively. (**B**) Quantification of AIF nuclear translocation is shown as the percentage of total AIF in cortical neurons. (**C**) NMDA (500 μM for 5 min) induced neurotoxicity. Results are presented as means ± S.E.M. for at least five independent experiments. ***$P<0.001$.

selectivity of the anti-AIF antibodies. The immunolabelling is corroborated by an almost complete loss of mitochondrial labelling in the Hq mice neocortex. Thus the immunogold results support the concept that AIF is associated with both the inner membrane and outer membrane, which is partly consistent with previous studies reporting AIF association with the inner mitochondrial membrane and intermembrane space (Arnoult et al., 2002).

The potential topology of AIF using tagged AIF peptides showed that mature AIF is an integral inner membrane protein with its N-terminal portion in the matrix and the bulk of the C-terminus in the inner membrane space (Otera et al., 2005). However, the GFP (green fluorescent protein)-tagged AIF carrying an extra 27 kDa peptide and forced over-expression of AIF or AIF peptide fragments may localize and behave differently from endogenous protein (Otera et al., 2005). The advantage and contribution of the present report is studying the localization of endogenous AIF using extensive biochemical approaches in combination with morphometric analysis in rodent brain tissues. Notably, AIF is proposed as an integral inner membrane protein after alkaline treatment at pH 10.5 (Otera et al., 2005), rather than pH 11.5, although pH 11.5 is the typical protocol for sodium carbonate treatment to distinguish between integral and peripheral membrane proteins (Fujiki et al., 1982). Peripheral membrane proteins are readily stripped by sodium carbonate treatment, whereas integral membrane proteins are solubilized only by the conditions disrupting the lipid bilayer. At pH 11.5, we recover AIF in the supernatant fraction.

Although the mechanisms by which the outer mitochondrial membrane is permeabilized remain contentious, the outer membrane has been regarded as the barrier containing dangerous pro-apoptotic proteins in the intermembrane space of mitochondria. The outer membrane theoretically needs to be broken or permeabilized for the release of pro-apoptotic molecules to activate downstream cell-death cascades. However, localization of AIF on the cytosolic surface of mitochondria would not require membrane permeabilization and may require other mechanisms for its release. Consistent with this notion, we observed that in the first phase of parthanatos approx. 14–20% of AIF is released from mitochondria in the first 2 h as the 62 kDa uncleaved form in the present study, as well as previous studies (Andrabi et al., 2006; Yu et al., 2006; Wang et al., 2009b).

The results of the present study clearly show that AIF is localized in two different compartments of brain mitochondria. Approx. 70% of AIF resides in the mitochondrial intermembrane space, and 30% of AIF associates with the mitochondrial outer membrane, which has not been reported previously, thus adding new insights into AIF function. There is precedence for integral mitochondrial proteins to exhibit localization to the mitochondrial outer membrane. For instance, yeast fumarase undergoes retrograde movement after mitochondrial import to reside, in part, on the mitochondrial outer membrane (Knox et al., 1998). Thus the biological mechanism of AIF localization on the mitochondrial outer membrane might involve a similar retrograde movement of the partially processed AIF back through the translocation pore (Knox et al., 1998). Alternatively NADH:cytochrome b_5 reductase exists in two different submitochondrial compartments via incomplete arrest in the outer membrane (Hahne et al., 1994). The arrest of a portion of the AIF precursor protein on the mitochondrial outer membrane where it is processed to mature AIF by interacting with mitochondrial protein import machinery may account for the outer mitochondrial membrane localization (Hahne et al., 1994). Further investigation is required to reveal the mechanism for the dual localization of AIF.

In summary, in the present study we find that there is a small, but significant, fraction of AIF associated with the outer mitochondrial membrane on the cytosolic side. Multiple biochemical approaches and ultrastructural analysis by electron microscopy detect endogenous AIF associated with the inner and outer membrane of mitochondria in the brain tissues. Dual localization of AIF may shed light into understanding the release mechanisms in parthanatos.

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