Cyclophilin A participates in the nuclear translocation of apoptosis-inducing factor in neurons after cerebral hypoxia-ischemia

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Upon cerebral hypoxia-ischemia (HI), apoptosis-inducing factor (AIF) can move from mitochondria to nuclei, participate in chromatinolysis, and contribute to the execution of cell death. Previous work (Cande, C., N. Vahsen, I. Kouranti, E. Schmitt, E. Daugas, C. Spahr, J. Luban, R.T. Kroemer, F. Giordanetto, C. Garrido, et al. 2004. Oncogene. 23:1514–1521) performed in vitro suggests that AIF must interact with cyclophilin A (CypA) to form a proapoptotic DNA degradation complex. We addressed the question as to whether elimination of CypA may afford neuroprotection in vivo. 9-d-old wild-type (WT), CypA+/−, or CypA−/− mice were subjected to unilateral cerebral HI. The infarct volume after HI was reduced by 47% (P = 0.0089) in CypA−/− mice compared with their WT littermates. Importantly, CypA−/− neurons failed to manifest the HI-induced nuclear translocation of AIF that was observed in WT neurons. Conversely, CypA accumulated within the nuclei of damaged neurons after HI, and this nuclear translocation of CypA was suppressed in AIF-deficient harlequin mice. Immunoprecipitation of AIF revealed coprecipitation of CypA, but only in injured, ischemic tissue. Surface plasmon resonance revealed direct molecular interactions between recombinant AIF and CypA. These data indicate that the lethal translocation of AIF to the nucleus requires interaction with CypA, suggesting a model in which two proteins that normally reside in separate cytoplasmic compartments acquire novel properties when moving together to the nucleus.

Cyclophilins constitute a family of phylogenetically conserved proteins found in prokaryotes as well as in humans. Cyclophilins have peptidyl-prolyl isomerase activity in vitro (1), indicating that they influence the conformation of proteins in cells, but their functions are largely unknown (2). The founding member of the cyclophilin family (15 members in humans), cyclophilin A (CypA), is an abundant, ubiquitously expressed protein originally discovered as an intracellular ligand of the immunosuppressive drug cyclosporin A (CsA) (3). CypA−/− embryonic stem cells grow normally and differentiate into hematopoietic precursor cells in vitro, indicating that CypA is not essential for mammalian cell viability (4). CypA−/− mice appear phenotypically normal, but with reduced fertility (unpublished data), and are resistant to immunosuppression by CsA (5). Immunosuppression is mediated by the CypA–CsA complex that inhibits calcineurin, a phosphatase crucial for T cell activation (6). CypA expression is particularly high in the brain, in neurons, where it is expressed mainly in the cytoplasm but also in the nucleus (7). Pioneering work by Montague et al. revealed that CypA possesses a latent, apoptosis-related DNase activity (8, 9). CypA has been
demonstrated to participate in excitotoxin-induced apoptosis (10) and to interact with a limited number of proteins, including dynemin (11), the antioxidant protein AopI (12), the nuclear pore protein Nup358 (13), and apoptosis-inducing factor (AIF) (14).

CypA cooperates with AIF during apoptosis-associated chromatinolysis in vitro (14). Jurkat cells lacking CypA expression are relatively resistant to AIF-induced cell death (14). Similarly, disruption of the CypA homologue CPR1 in yeast cells abrogated cell death induced by overexpression of AIF (15). In vitro, recombinant AIF and recombinant CypA together have a higher DNase activity than each of the proteins alone (14). Mutant CypA that lacks peptidyl-prolyl isomerase activity still cooperates with AIF to mediate chromatinolysis, and the proapoptotic cooperation of CypA and AIF was not inhibited by CsA (14), indicating that CsA is not a pharmacological tool to investigate the contribution of CypA and AIF to cell death.

We and others have demonstrated that AIF plays an important role in neuronal death after focal ischemia in the adult rodent brain (17–20) and after neonatal hypoxia-ischemia (HI) (21, 22). AIF translocates from mitochondria to the nuclei of dying neurons, and inhibition of this translocation (18, 19) or reduction of AIF expression through the harlequin (Hq) mutation is neuroprotective (17, 22). Thus, AIF contributes to the pathogenesis of adult stroke and perinatal asphyxia. The aim of this study was to explore the involvement of CypA in brain injury induced by neonatal HI. We demonstrate that CypA and AIF functionally and physically interact in vivo in neonatal HI and in vitro using surface plasmon resonance, and that the CypA knockout still cooperates with AIF to mediate chromatinolysis, considering that the infarct cores are beyond rescue and that neuroprotective strategies can salvage only penumbral areas. The degree of protection is in the same range as that observed for mice with relative AIF deficiency, the so-called Hq mice (22). A functional connection between AIF and CypA was recently found in vitro, demonstrating that AIF and CypA bind to each other upon induction of apoptosis in Jurkat cells and that the AIF–CypA complex, but neither of the individual constituents alone, could produce nonspecific, large-scale DNA degradation (14).

We next examined the mechanisms underlying the neuroprotective effect of CypA deficiency. No CypA protein was detected in CypA−/− mice, and the level of CypA protein was reduced ~50% in CypA+/− compared with WT mice, as judged by immunoblotting (Fig. 2 A). CypA−/− mice were not phenotypically different from WT mice, and the brain morphology was not different between the two genotypes (unpublished data). The expression of neither the mitochondrial proapoptotic proteins AIF, Cyt c, and Smac, nor the mitochondrial oxidative stress–related proteins SOD2 and Trx2, were changed in CypA−/− nonischemic mice compared with CypA+/− or WT mice (Fig. 2 A). 24 h after HI, the mitochondrial proteins AIF, cyt c, Smac, and SOD2 were reduced in the mitochondrial fraction of the ipsilateral cortex compared with the contralateral side, but the decrease was the same in WT and CypA−/− mice (Fig. 2 B). Thus, CypA deficiency does not inhibit the mitochondrial release of cell-death effectors. The nonmitochondrial oxidative stress–related proteins catalase and SOD1 were not changed at 24 h after HI (Fig. 2 C). Hsp70, a chaperone known to counteract the proapoptotic effects of AIF, was not different between WT and CypA−/− mice (Fig. 2 C). The fibrin breakdown products generated by calpain (145/150 kD) and caspase-3 activation (120 kD) were not different between WT and CypA−/− mice, nor was the calpain-induced degradation of Na+/Ca2+ exchanger 1 (NCX1; Fig. 2 C). Accordingly, the capacity to cleave caspase-3 (Fig. 2 D) and caspase-9 (not depicted) peptide substrates was not different in WT compared with CypA−/− mice in homogenates of normal control or postischemic cortical tissue 24 h after HI. Thus, CypA deficiency had no effect on the HI-induced activation of proteases, including calpains and caspases. The levels of AIF, Hsp70, or oxidative stress–related proteins were not different between WT and CypA−/− mice and, hence, cannot explain the AIF–related, protective effects observed in CypA−/− mice. The AIF deficiency in Hq mice reduces the antioxidant defense of neurons, thereby enhancing their susceptibility to oxidative stress (22, 24), but we could not find any indications of altered levels of antioxidative stress–related proteins in CypA−/− mice. Interestingly, caspase-3– and caspase-9–like activities increased after HI to a similar level in WT and CypA−/− mice despite the fact that WT mice developed brain infarcts that were almost twice as large as those from CypA−/− mice. This is in accordance with our previous finding that the neuroprotection afforded by the Hq mutation does not correlate with reduced caspase
mice carrying the Hq mutation, CypA staining in the nucleus was reduced (Fig. 3 C). These data suggest that CypA is required for the optimal nuclear translocation of AIF in damaged neurons and, vice versa, that AIF is needed for the nuclear translocation of CypA after HI. Accordingly, CypA coimmunoprecipitated with AIF in the ipsilateral, ischemic hemisphere but not in normal control tissue (Fig. 4 A). This suggested a direct physical interaction between AIF and CypA, and this was confirmed using surface plasmon resonance (Biacore). Recombinant AIF was captured by an antibody immobilized to the sensor surface, and recombinant CypA bound to and was released from the captured AIF with an apparent dissociation constant ($K_d$) of 1 μM (Fig. 4 B). Thus, direct physical interaction between AIF and CypA was demonstrated in vitro and in vivo after HI.

Double labeling of AIF and the DNA strand break marker Tdt-mediated dUTP-biotin nick-end labeling (TUNEL) revealed intense nuclear AIF immunoreactivity in TUNEL-positive cells from WT mice (Fig. 5 A) but reduced nuclear AIF immunoreactivity in TUNEL-positive CypA$^{-/-}$ neurons (Fig. 5 B). Similarly, TUNEL-positive nuclei in WT mice displayed strong CypA immunoreactivity (22), yet it stands in contrast with the notion that caspases, particularly caspase-3, are critically important for the development of brain injury in the immature brain (22, 25). This may suggest that caspase-independent apoptosis-related cell-death mechanisms are particularly important in determining cell fate after brain injury. Clearly, the CypA–AIF interaction plays a greater role in pathological neuronal death than in normal, developmentally regulated programmed cell death, because CypA$^{-/-}$ mice do not display any dysmorphic or teratogenic features (5).

### Functional and physical interaction of CypA and AIF after HI in neurons

In healthy neurons, CypA is localized both in the cytoplasm and nuclei, whereas AIF is confined to mitochondria. In WT mice, nuclear translocation of both AIF and CypA was evident in injured (pyknotic) neurons after HI, as judged by immunofluorescence stainings (Fig. 3 A). In a separate experiment, 93% of the cells with nuclear AIF were identified as neurons (NeuN positive; unpublished data). In CypA$^{-/-}$ mice, nuclear translocation of AIF could be detected in injured neurons, but the signal was very weak (Fig. 3 B) compared with WT mice (Fig. 3 A). Conversely, in AIF–deficient mice carrying the Hq mutation, CypA staining in the nucleus was reduced (Fig. 3 C). These data suggest that CypA is required for the optimal nuclear translocation of AIF in damaged neurons and, vice versa, that AIF is needed for the nuclear translocation of CypA after HI. Accordingly, CypA coimmunoprecipitated with AIF in the ipsilateral, ischemic hemisphere but not in normal control tissue (Fig. 4 A). This suggested a direct physical interaction between AIF and CypA, and this was confirmed using surface plasmon resonance (Biacore). Recombinant AIF was captured by an antibody immobilized to the sensor surface, and recombinant CypA bound to and was released from the captured AIF with an apparent dissociation constant ($K_d$) of 1 μM (Fig. 4 B). Thus, direct physical interaction between AIF and CypA was demonstrated in vitro and in vivo after HI.
immunoreactivity in TUNEL-positive nuclei (Fig. 5 D). The number of TUNEL-positive nuclei also positive for AIF or CypA was determined in WT, CypA−/−, and Hq mice 24 h after HI within the penumbra. Approximately 253 TUNEL-positive neurons per mm² displayed nuclear AIF staining in WT mice, whereas in CypA−/− mice the number of TUNEL-positive cells with AIF staining was reduced by 90.7% (P < 0.0001; Fig. 5 E). Similarly, the number of TUNEL/CypA double-positive neurons decreased by 79.4% in Hq compared with WT mice (P < 0.0004; Fig. 5 F). Furthermore, the total number of TUNEL-positive cells after HI was reduced to a greater extent in CypA−/− and Hq mice than would be expected from the degree of protection (Fig. 5, E and F).

These findings provide a possible mechanism whereby AIF could exert its proapoptotic effects when released from mitochondria. We anticipate that under normal conditions, AIF and CypA reside in separate cellular compartments, with AIF in the mitochondrial intermembrane space and CypA in the cytoplasm and nuclei. Thus, both proteins would be prevented from interacting under normal conditions but can bind to each other upon induction of cell-death signaling and mitochondrial membrane permeabilization. The domain of the AIF protein that interacts with CypA is separate from its HSP70 interaction domain and from its DNA binding sites (14). The domain of CypA that binds to AIF is separate from its catalytic domain and its binding site for CsA (14). Previous studies could not conclusively determine in which subcellular compartment the AIF–CypA interaction occurred, but it was assumed that AIF underwent electrostatic interaction with DNA (26) and then tethered CypA to chromatin (14). Our data suggest that the CypA–AIF interaction occurs before or when nuclear translocation begins, in the cytosol.
This suggests a direct physical interaction between AIF and CypA, and this was confirmed using coimmunoprecipitation and surface plasmon resonance technology. Presumably, the physical interaction between CypA and AIF facilitates nuclear translocation of the two proteins as a complex.

In summary, this is the first study to our knowledge demonstrating that elimination of CypA affords neuroprotection in vivo. It has been demonstrated in vitro that AIF and CypA cooperate in caspase-independent apoptosis-related DNA fragmentation. Our in vivo data support and extend this cooperative mechanism. Until now, the only possibility to inhibit the lethal action of AIF in HI was to prevent mitochondrial membrane permeabilization, e.g., by targeting the antiapoptotic Bcl-2 family protein Bcl-XL to neurons (20), or to prevent the translocation of AIF to the nucleus by overexpressing transgenic HSP70 in neurons (18). Our data demonstrate yet another possible strategy for inhibiting the action of AIF, namely by generating pharmacological inhibitors of the CypA–AIF interaction. This strategy constitutes a challenging endeavor for future research.

This is based on the observations that nuclear translocation of AIF was reduced (by ~90%) in injured neurons in CypA−/− mice and, conversely, that CypA translocation to nuclei was reduced (by ~80%) in AIF-deficient Hq mice. Conclusively, these data suggest that CypA is required for efficient nuclear translocation of AIF in damaged neurons and, conversely, that AIF is needed for the nuclear translocation of CypA after HI.
Figure 5. Obligate cotranslocation of AIF and CypA into the nuclei of damaged neurons. DNA strand breaks in the cortex 3 or 24 h after HI were identified using TUNEL labeling (green) and correlated with AIF (A and B, red) or CypA (C and D, red) immunolabeling. Nuclear morphology was evaluated with HOECHST 33342 (blue). (A) A TUNEL-positive neuron (arrow, green) displaying strong nuclear AIF staining (red) and condensed chromatin (blue), unlike the noninjured, TUNEL-negative neurons. (B) In CypA−/− mice nuclear AIF staining (arrow, red) was weak in the TUNEL-positive neurons (green) with condensed chromatin (blue). (C) A TUNEL-positive neuron (arrow, green) displaying strong nuclear CypA staining (red) and condensed chromatin (blue), unlike the noninjured, TUNEL-negative neurons. Bar, 10 μm. (D) In AIF-deficient Hq mice, TUNEL-positive neurons (arrow, green) with condensed nuclear morphology (blue) displayed weak nuclear CypA staining (red). (E) Quantification of TUNEL-positive cells with AIF-positive nuclei in the cortical penumbra 24 h after HI. WT brains contained 276 ± 44 TUNEL-positive neurons per mm² 24 h after HI, whereas CypA−/− mice displayed only 78 ± 10 neurons per mm² (**, P = 0.0003). The density of cells positive for both TUNEL and AIF was 253 ± 40 cells per mm² in WT and 23 ± 6 cells per mm² in CypA−/− mice (n = 6 per group; ***, P < 0.0001). (F) Quantification of TUNEL-positive cells with CypA-positive nuclei in the cortical penumbra 24 h after HI. WT mice exhibited 170 ± 31 TUNEL-positive neurons per mm² 24 h after HI, whereas this value amounted to 64 ± 8 neurons per mm² in Hq mice (**, P = 0.0011). The density of cells positive for both TUNEL and CypA was 150 ± 28 cells per mm² in WT and 29 ± 2 cells per mm² in Hq mice (n = 6 per group; ***, P < 0.0004).
MATERIALS AND METHODS

HI. CypA+/+ mice (S) and their heterozygous (CypA+/-) or WT littermates of either sex were subjected to unilateral HI on postnatal day 9 (P9), essentially according to the Rice-Vannucci model (28). AIF-deficient Hq male mice (24) and their WT littermates were subjected to the same procedure. In brief, mice were anesthetized with isoflurane, and the left common carotid artery was cut between double ligatures of proline sutures. After surgery, the pups were allowed to recover for 1–1.5 h with the dam and were then placed in a chamber perfused with a humidified gas mixture (10% oxygen in nitrogen) for 45 min. After hypoxic exposure, the pups were returned to their biological dams until they were killed. Interesting differences between neuronal mechanisms of injury in neonatal male and female brains have been observed, including more pronounced nuclear translocation of AIF during early reperfusion (P < 0.05) (27). We did not find any significant differences between males and females regarding the injury observed or the frequency of neurons with nuclear AIF (unpublished data), as expected at the particular time points of evaluation shown in the figures (22, 27), so both male and female CypA+/-, CypA+/-, and WT pups were included in this study. All animal experimental protocols were approved by the Götete committee of the Swedish Animal Welfare Agency (application nos. 94–2003 and 184–2003).

Genotyping. Hq genotyping was previously described (22). For CypA mice genotyping, the following primers were used: CypA common, 5'-GCAATTCTGTTGATGTAGCCACGGC-3'; CypA WT, 5'-CACCTGTGGAGCACCACTGCCCACC-3'; and CypA mutant, 5'-CCTGATCGAACAAGCCCGGCTTCC-3'. CypA +/- mice were identified by the presence of a single 520-bp DNA band. WT mice were identified by a single 200-bp band.

Immunohistochemistry. Mice were deeply anesthetized with 50 mg/ml phenobarbital and perfusion fixed with 5% formaldehyde. Brains were dehydrated with xylene and graded ethanol, paraffin embedded, and serial cut into 5-μm coronal sections. Sections were deparaffinized, and antigen retrieval was performed by boiling them in 10 mM sodium citrate buffer (pH 6) for 10 min. Nonspecific binding was blocked for 30 min with 4% horse serum in PBS. 4% donkey serum in PBS. 2 Anti-AIF (0.05 μg/ml in 10 mM sodium acetate, pH 5) was amine-coupled to the antibody surface, because the dissociation rate was very slow. 75 nM CypA protein (Affinity BioReagents, Inc.) in running buffer with 1 mM nicotinamide adenine dinucleotide phosphate (NADPH) was passed over the surface, resulting in nucleotide phosphate (NADPH) was passed over the surface, resulting in immobilized immune complexes were pelleted by centrifugation at 3,000 g for 5 min at 4°C and washed four times with 0.5 ml homogenizing buffer. The bound proteins were eluted by heating in NuPAGE LDS 4X sample buffer for 10 min. Immunoprecipitated proteins were subjected to immunoblotting and examined for AIF and CypA immunoreactivity.

Western blot analysis. Homogenates and cellular fractions from parietal cortex (harboring both injured and intact tissue) were prepared as previously described (22). The samples were run on 4–12% NuPAGE Bis-Tris gels (Novex) and transferred to reinforced nitrocellulose membranes (Schleicher & Schuell). The following primary antibodies were used: anti-actin (1:200; Sigma-Aldrich), 0.2 μg/ml anti-AIF, 5 μg/ml antitubulin (clone 11A; Lab Frontier), anti-CypA (1:4,000), anti-cytochrome c (1:500; clone 7H8.2C12; BD Biosciences), 0.2 μg/ml antifodrin (clone A16; BIO-MOL Research Laboratories, Inc.), 0.5 μg/ml anti-heat-shock protein 70 (HSP 70; Santa Cruz Biotechnology, Inc.), 0.7 μg/ml anti-Smad (Santa Cruz Biotechnology, Inc.), 0.5 μg/ml anti-superoxide dismutase I (Cu/Zn-SOD/SOD1; Lab Frontier), 0.5 μg/ml anti-superoxide dismutase II (Mn-SOD/SOD2; clone 2A1; Lab Frontier), 1 μg/ml anti-thioredoxin 2 (Trx2; Lab Frontier), and 0.25 μg/ml anti-NCX1 (1:800; Santa Cruz Biotechnology, Inc.). Peroxidase-labeled secondary antibodies (1:2,000 goat anti–rabbit, 1:2,000 horse anti–goat, or 1:4,000 horse anti–mouse) were obtained from Vector Laboratories. Immunoreactive species were visualized using a substrate (Super Signal Western Dura; Pierce Chemical Co.) and a cooled CCD camera (LAS 3000; Fuji).

Surface plasmon resonance. CypA interaction with AIF was explored with the surface plasmon resonance technique, using a BIACORE 3000 (Biacore AB). The BIACORE HBS-EP buffer (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, and 0.01% polysorbate P20, pH 7.4) was used as a running buffer at 5 μl/min and 25°C in all experiments. A goat polyclonal anti-AIF (sc-9416; Santa Cruz Biotechnology, Inc.) and incubation overnight at 4°C. Immune complexes were sequestered through the addition of 50 μl of Pansorbin cells and incubation for an additional 2 h at 20°C. The resulting immuno globally complexed nucleic acid was pelleted by centrifugation at 3,000 g for 5 min at 4°C and washed four times with 0.5 ml homogenizing buffer. The bound proteins were eluted by heating in NuPAGE LDS 4X sample buffer for 10 min. Immunoprecipitated proteins were subjected to immunoblotting and examined for AIF and CypA immunoreactivity.

Injury evaluation. Brain injury was evaluated by neuropathological scoring as well as calculating infarct volume and the volume of total tissue loss at 72 h after HI (22). Sections were stained for MAP2 and scored by an observer blinded to the genotype of the mice. The total score (0–22) was the sum of the scores for all four regions (cortex, hippocampus, striatum, and thalamus) (29). The infarct areas (MAP2 negative) were measured using Micro-Imaging (Olympus). Total tissue loss was calculated as the MAP2-positive areas in the contralateral hemisphere minus the MAP2-positive areas in the ipsilateral hemisphere (29). Immunoprecipitation. AIF was immunoprecipitated from control tissue or ipsilateral (ischemic) hemispheres 3 h after HI. Homogenate samples containing equal amounts of protein were precleaned with goat IgG and PANSORBIN cells (Calbiochem), followed by the addition of 1 μg anti-AIF (sc-9416; Santa Cruz Biotechnology, Inc.) and incubation overnight at 4°C. Immune complexes were sequestered through the addition of 50 μl of Pansorbin cells and incubation for an additional 2 h at 20°C. The resulting immuno complexes were pelleted by centrifugation at 3,000 g for 5 min at 4°C and washed four times with 0.5 ml homogenizing buffer. The bound proteins were eluted by heating in NuPAGE LDS 4X sample buffer for 10 min. Immunoprecipitated proteins were subjected to immunoblotting and examined for AIF and CypA immunoreactivity.
bound AIF was reached. A dissociation equilibrium constant for AIF and CyPA of ~4 μM was determined using BLAeval software (Blucore AB), assuming mass transport.

**Statistics.** All data were expressed as mean ± SEM. The Student’s t test was used when comparing two groups, whereas analysis of variance with Fisher’s post-hoc test was used when comparing three or more groups. The Mann-Whitney U test was used when comparing the ratios of double-positive cells.

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