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RESEARCH ARTICLE

Cyclophilin A is a mitochondrial factor that forms complexes with p23 – correlative evidence for an anti-apoptotic action

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ABSTRACT

Cyclophilin A (CyPA, also known as PPIA) is an abundant and ubiquitously expressed protein belonging to the immunophilin family, which has intrinsic peptidyl-prolyl-(*cis*/*trans*)-isomerase enzymatic activity. CyPA mediates immunosuppressive action of the cyclic undecapeptide cyclosporine A and is also involved in multiple cellular processes, such as protein folding, intracellular trafficking, signal transduction and transcriptional regulation. CyPA is abundantly expressed in cancer cells, and, owing to its chaperone nature, its expression is induced upon the onset of stress. In this study, we demonstrated that a significant pool of this immunophilin is primarily an intramitochondrial factor that migrates to the nucleus when cells are stimulated with stressors. CyPA shows anti-apoptotic action per se and the capability of forming ternary complexes with cytochrome c and the small acidic co-chaperone p23, the latter interaction being independent of the usual association of p23 with the heat-shock protein of 90 kDa, Hsp90. These CyPA•p23 complexes enhance the anti-apoptotic response of the cell, suggesting that both proteins form a functional unit, the high level of expression of which plays a significant role in cell survival.

KEY WORDS: Cyclophilin A, Immunophilin, p23, Mitochondria, Apoptosis, Trafficking

INTRODUCTION

Cyclophilin A (CyPA) is an abundant protein belonging to the immunophilin (IMM) family. As such, it is characterized by the presence of a signature domain of the family, the peptidyl-prolyl isomerase (PPIase) domain, which is capable of catalysing the *cis*↔*trans* isomerization of peptidyl-prolyl bonds (Nigro et al., 2013; Zgajnar et al., 2019). CyPA was originally isolated from bovine thymocytes and identified as the cytosolic receptor for the immunosuppressive cyclic undecapeptide cyclosporin A, such that the formation of a CyPA•drug complex impairs the transcription of genes related to the immune response and prevents proliferation of T cells (Handschumacher et al., 1984).

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CyPA is ubiquitously expressed in the cell, and can also be secreted to the medium in response to inflammatory stimuli, such as hypoxia, infection and oxidative stress, among others (Nigro et al., 2013). CyPA shows pleiotropic actions that comprise protein folding, trafficking, cell activation, cardiovascular effects and chemotaxis, and is related to atherosclerosis, diabetes, viral infections, drug resistance, cancer, neurodegenerative diseases, ageing, etc. (Dawar et al., 2017; de Wilde et al., 2018; Nigro et al., 2013; Xue et al., 2018). Importantly, the secreted form of CyPA shows autocrine and paracrine properties by binding to the CD147 (also known as BSG) cell surface receptor (Pushkarsky et al., 2001), favouring the release of interleukin-6 (IL6) from inflammatory cells (Payeli et al., 2008), chemotaxis (Yurchenko et al., 2010), upregulation and secretion of MMP9 (Yang et al., 2008), glioma progression (Xu et al., 2020), multiple myeloma development (Zhu et al., 2015), activation of prosurvival neuronal pathways (Boulos et al., 2007; Yurchenko et al., 2002), etc.

Even though CyPA may play relevant roles during protein folding and the induction of conformational changes of various proteins thanks to its PPIase activity, it also functions as a standard molecular chaperone modulating protein-protein interactions regardless of its intrinsic enzymatic activity (Rein, 2020). Thus, CyPA has been related to the active cytosolic mechanism of retrotransport of soluble proteins via dynein in a PPIase domain-dependent and enzymatic activity-independent manner (Galigniana et al., 2004). Several studies have also demonstrated that CyPA induces leukocyte chemotaxis through direct binding to the ectodomain of receptor CD147 in a PPIase-independent fashion (Song et al., 2011). Similarly, CyPA impairs the early stage of influenza A virus replication by simple protein-protein interaction promoting the degradation of the virus matrix protein M1 (Mahesutihan et al., 2018). It has also been postulated to be a potential biomarker and mediator in cardiovascular diseases, and a key modulator of the vascular architecture (Satoh et al., 2010).

Inasmuch as many PPIases are also molecular chaperones, it has recently been discussed that the association of this family of proteins to client factors can influence the energy landscape by destabilization of intermediates and unfolding activity (Galigniana, 2020; Rein, 2020). Rein (2020) has proposed the challenging hypothesis that these events may be totally independent of the enzymatic activity depending on the specific client protein. In this regard, it should be pointed out that molecular chaperones often act in a sequential manner. For example, those chaperones associated with steroid receptors and protein kinases are usually transferred from the Hsp70 folding machinery to the Hsp90-based folding platform (Pratt et al., 2004b), which replaces intermediate complexes containing the co-chaperone Hop/p60 by a high molecular weight IMM. The entire heterocomplex is finally stabilized by the small acidic Hsp90-binding co-chaperone, p23 (Pratt et al., 2004a). It is accepted that the overexpression of Hsp70 or Hsp90 inhibits apoptosis and prevents caspase activation in many cellular models upon accumulation of misfolded proteins, oxidative stress or DNA damage (Lanneau et al., 2008; Mosser and Morimoto, 2004).

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The recruitment or overexpression of the co-chaperone p23 should consequently contribute to this outcome. It has been recently reported that the skin sections of $p23^{-/-}$ mice are clearly thinner than the skin of wild-type animals and show a high percentage of apoptotic cells (as demonstrated by TUNEL assay), and also that BrdU staining of cell nuclei is augmented, indicating increased cell proliferation, perhaps to partially counterbalance the high apoptotic rate (Madon-Simon et al., 2017). Because the phenotype of p23-null embryos is like the one observed when the glucocorticoid receptor function is abolished, it was speculated that the phenotype occurred primarily because of the impact p23 has on this particular transcription factor.

CyPA has also been related to the mechanism of apoptosis by favouring the nuclear translocation of apoptosis-inducing factor (AIF) (Zhu et al., 2007). *In vitro*, recombinant AIF and CyPA cooperate in the degradation of plasmid DNA and induce DNA loss in purified nuclei. However, CyPA downregulation does not arrest nuclear translocation of AIF, although it reduces DNA damage (Artus et al., 2010). In an alternative model, it was shown that one of the first responses to oxidative stress involves the rapid accumulation of CypA in the nucleus some hours before apoptosis is triggered (Doti et al., 2014). Targeting of CypA with synthetic peptides to inhibit the AIF-mediated neuronal loss was also used as a valuable strategy to protect cells (Rodriguez et al., 2020). This raises the possibility that endogenous factors capable of interacting with CyPA may also function as protective factors against apoptosis, a seductive hypothesis that is examined in this study.

On the other hand, CyPA has been shown to be implicated in essential neuronal functions, such as axonal transport, synaptic vesicle assembly, neuroprotective roles against abnormal protein aggregation (Avramut and Achim, 2003), and as a necessary factor related to the BDNF neuroprotective action (Jeon et al., 2008; Lin et al., 2014). Also, it has been suggested that CyPA may play a neuroprotective role against both oxidative stress (Doyle et al., 1999) and ischemic events (Boulos et al., 2007). Moreover, it has also been reported that the direct injection of purified CyPA exerts protective roles after brain injury (Redell et al., 2007). In other words, CyPA influences a wide variety of biochemical and pathophysiological pathways in several models of disease, often in an opposite manner, its effects being related to the type of complexes CyPA participates.

In preliminary confocal microscopy studies, we noted that CyPA shows a subcellular fluorescent pattern quite compatible with a primary mitochondrial localization. During these observations, a strong colocalization of CyPA with the Hsp90-binding co-chaperone p23 was noted. In view of the reports referenced above describing how both proteins may individually participate in apoptotic pathways, we hypothesize that it could be entirely possible that CyPA is a mitochondrial factor that impacts on apoptosis itself, and that both chaperones CyPA and p23 may belong to complexes that could influence cell survival. In this study it is demonstrated that CyPA is a novel mitochondrial factor with anti-apoptotic properties that are enhanced by the interaction of CyPA with the co-chaperone p23 in an Hsp90-independent manner.

RESULTS

CyPA is a mitochondrial factor

Confocal microscopy images of several cell types showed that CyPA (green) exhibits a profile compatible with mitochondrial localization Fig. 1A. Colocalization of the IMM with the organelle was evidenced by indirect immunofluorescence using the The Scripps Research Institute (TSRI) rabbit IgG against CyPA and the specific mitochondrial fluorescent probe MitoTracker (red). Identical colocalization images were also seen when three different anti-CyPA antibodies from diverse suppliers were assayed, i.e. goat polyclonal (Invitrogen, PA5-18463), which is shown in Fig. 1B, and two rabbit polyclonal antibodies purchased from Invitrogen (PA1-025) and from GeneTex (GTX104698), the latter shown in Fig. 1C. Similarly, CyPA co-stained with mitochondrial proteins, such as cytochrome c (Cyt c), cytochrome c oxidase subunit IV (COX-IV) and Tom-20 (data not shown). Fig. 1B shows the colour profiles for CyPA and MitoTracker, indicating a high percentage of signal overlapping, whereas Fig. 1C shows the true colocalization of both signals after subtracting the non-specific background by applying a deconvolution plug-in software (see black and white panel).

The amino acid sequence of CyPA does not show a known mitochondrial localization signal that may justify its localization. Therefore, it was worth analysing the potential requirement of the intrinsic PPIase activity of CyPA for such subcellular localization. Fig. 2A shows that the PPIase activity is not required as the inactive point mutant H126Q showed the same colocalization with the mitochondrial probe as the endogenous IMM.

To confirm the mitochondrial localization of CyPA suggested by confocal microscopy images, mitochondria were purified by biochemical fractionation from both 3T3-L1 cells (Fig. 2B) and rat liver tissue (Fig. 2C). Lamin B and NuMa were used as specific protein markers of the nuclear fraction, COX-IV as a mitochondrial marker and calnexin (CNX, also known as CANX) as a microsomal marker in the case of liver extracts, which underwent an additional 100,000 ***g*** centrifugation to clarify the cytosolic extract due to the high content of low-density lipids (not present in 3T3-L1 extracts). CyPA was detected in all the fractions, including mitochondria. Inasmuch as CyPD (also known as PPIase-D) is a mitochondrial IMM that shows high homology with CyPA and regulates the calcium efflux through the permeability transition pore (Mehta, 2018), a control experiment was performed to demonstrate that the anti-CyPA antibody cannot recognize mitochondrial CyPD. Fig. 2D shows western blots for both proteins from purified mitochondria that have been resolved in a highly resolutive 15% SDS-PAGE gel. Clearly, the anti-CyPA antibody does not cross-react with CyPD.

To determine whether CyPA is within mitochondria rather than associated with the outer membrane of the organelle, purified mitochondria form 3T3-L1 fibroblasts were treated for 15 min at 4°C with proteinase K (5 ng/ml in lines 2 and 5, or 50 ng/ml in lines 3 and 6). Proteins were then resolved by western blot (Fig. 2E). Lanes 2 and 3 show that the presence of CyPA in the mitochondrial pellet remained unchanged after the controlled digestion with the protease. However, the pretreatment of mitochondria with 1% SDS for 10 min degraded the IMM in a protease concentration-dependent manner. This indicates that CyPA is indeed an intramitochondrial factor rather than a protein associated with the outer membrane of the organelle.

CyPA migrates to the nucleus and shows anti-apoptotic properties

Interestingly, CyPA increases its nuclear localization upon the onset of any type of stress. Therefore, it is important to point out that the notorious mitochondrial localization evidenced in fresh cell cultures grown under optimal conditions is lost along the number of passages of the culture when it ages, as well as when even fresh cells become confluent or the medium is not conveniently replaced. These casual observations led us to analyse the subcellular localization of CyPA under several situations of stress (i.e. heat-shock, serum starvation, oxidants, UV light, etc.). As a representative observation among a number of assayed stress situations, Fig. 3A shows the nuclear translocation of CyPA after the addition to the medium of either

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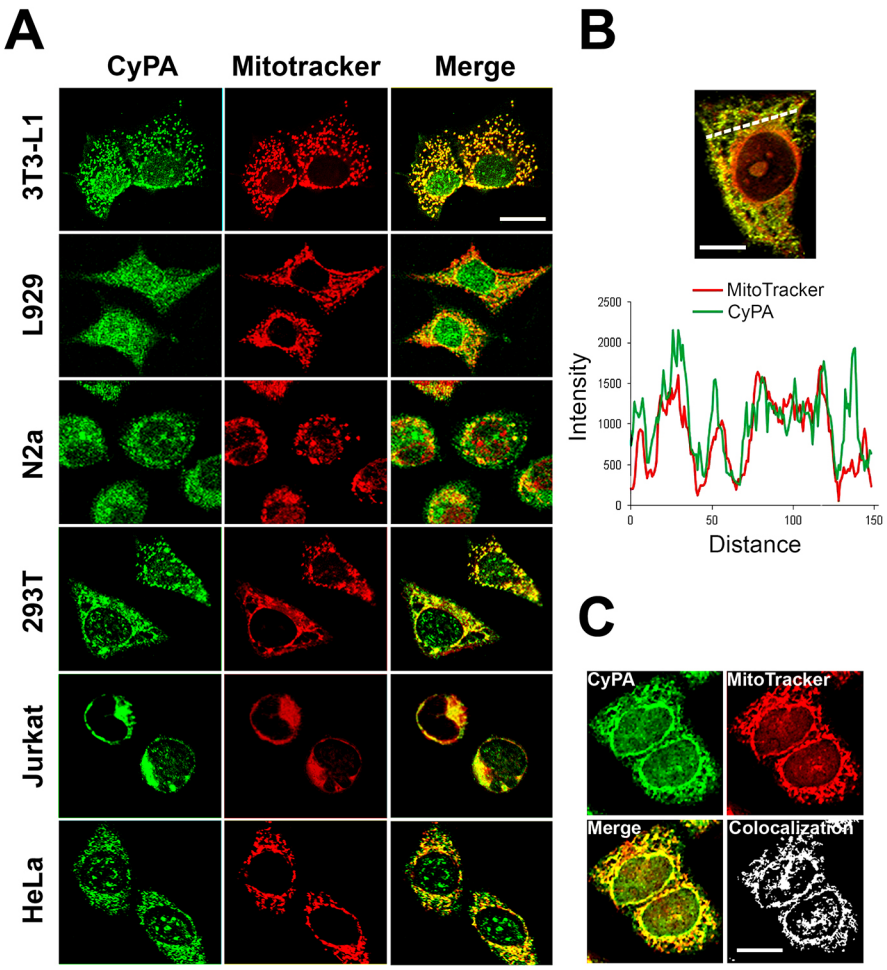

Fig. 1. CyPA is a mitochondrial protein. (A) Indirect immunofluorescence images by confocal microscopy show the colocalization of endogenous CyPA (green) with the mitochondrial marker MitoTracker (red) in several cell types. (B) Overlapping of colour profiles for CyPA staining (green) and mitochondria staining (red) along the dashed-white line in the HeLa cell shown above the plot. (C) Colocalization mask of a *z*-stack image for 3T3-L1 cells, showing the true colocalization of CyPA with mitochondria. Scale bars: 10 µm.

aggressor, hydrogen peroxide or staurosporine. Such nuclear translocation shows an average half-life of 2 h for most assayed stimuli, including these two. Therefore, 4 h after the onset of stress, CyPA becomes almost entirely nuclear in all cell types assayed.

We have previously described similar mitochondrial-nuclear translocation for another immunophilin, FKBP51, which also shows anti-apoptotic properties associated with such subcellular relocalization (Gallo et al., 2011). Therefore, the viability of cells overexpressing CyPA or not was analysed after treatment with hydrogen peroxide or staurosporine. The plots shown on the right side of Fig. 3A clearly evidence the protective effect of CyPA along the entire range of concentrations of the pro-apoptotic stimulus. Similar conclusions can be reached after analysing the cells with acridine orange/ethidium bromide in stauroporine-treated cells (Fig. 3B), the procaspase-3 cleavage after cell exposure to H_2O_2 (Fig. 3C) or cell staining with JC-1, a mitochondrial membrane potential marker, in etoposide-induced apoptosis conditions (Fig. 3D).

Taken together, all these findings demonstrate that CyPA is a mitochondrial factor that undergoes trafficking towards the nucleus and preserves cell viability. It should be noted that CyPA is also a molecular chaperone and its upregulation has been reported under several situations of stress, as well as in several types of cancers (melanoma, small cell lung cancer, pancreatic cancer, breast cancer, colorectal cancer, squamous cell carcinoma, etc.). A correlation between CypA overexpression and malignant transformation has also been established (Lee, 2010; Nigro et al., 2013).

CyPA and cytochrome c release from mitochondria by oxidative stress

The subcellular localization of both CyPA and Cyt c was evidenced in cells stimulated with H_2O_2. HeLa cells were assayed because they have a good nuclear/cytoplasmic ratio. Fig. 4A shows that Cyt c localized entirely in the mitochondria of unstimulated cells along with a significant fraction of CyPA, whereas after 2 h of cell exposure to H_2O_2, a minimal fraction of each protein was recovered in the organelle, suggesting that the mitochondrial export rate was quite similar for both factors. Although both proteins can be detected in the cytoplasm (presumably, in transit as after 4 h both proteins are nuclear) a significant nuclear accumulation is already evident.

After 4 h of treatment with H_2O_2, most CyPA and Cyt c are nuclear, although a minor fraction of each endogenous factor can still be recovered in the cytosol. This can be seen in the indirect immunofluorescence images and the western blot of the subcellular fractionation shown in Fig. 4B. Interestingly, Fig. 4C demonstrates that overexpression of HA-CyPA results in more Cyt c being retained in the cytoplasm under identical conditions, suggesting that CyPA and Cyt c may interact physically, the biological consequence being the prevention of the nuclear translocation of Cyt c. If true, this may be part of the anti-apoptotic mechanism of action of CyPA. Note that transfected cells in Fig. 4C show the same punctuate mitochondrial pattern for the exogenous HA-CyPA as Fig. 4B shows for endogenous CyPA.

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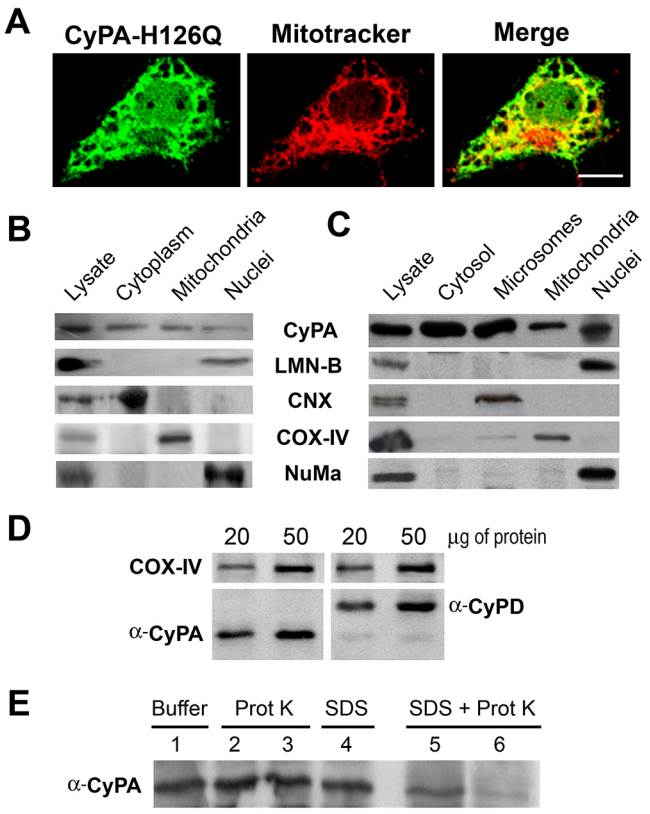

Fig. 2. CyPA is intramitochondrial. (A) 3T3-L1 cells were transfected with the PPIase inactive mutant pBEX1-HA-hCyPAH126Q. The subcellular localization of the mutant was visualized using an anti-HA primary antibody, followed by a secondary antibody labelled with Alexa Fluor 488. Mitochondria were stained with MitoTracker Deep Red-633. Scale bar: 10 µm. (B,C) To confirm the mitochondrial localization of endogenous CyPA, a subcellular fractionation of 3T3-L1 cell extracts (B) or rat liver tissue (C) was performed. The following protein markers were used: lamin B (LMN-B) and nuclear mitotic apparatus protein (NuMa) for nuclei; COX-IV for mitochondria; and CNX for endoplasmic reticulum and vesicle-like artifacts. (D) Total proteins (20 µg and 50 µg) from purified mitochondria of 3T3-L1 cells were resolved in a 15% SDS-PAGE gel, followed by western blotting for CyPA (left hand membrane), CyPD (right hand membrane) and COX-IV as loading control (top). (E) Isolated mitochondria from rat liver were incubated with 1% SDS (lanes 4, 5 and 6) and/or proteinase K at 5 ng/ml (lanes 2 and 5) or 50 ng/ml (lanes 3 and 6). CyPA was resolved by western blot.

CyPA and p23 colocalization

The small acidic co-chaperone p23 is usually associated with the molecular chaperone Hsp90 in most heterocomplexes. Nonetheless, p23 has also Hsp90-independent chaperone activity that can prevent protein aggregation and maintain client proteins in a folding-competent state (Freeman et al., 1996). As stated before, molecular chaperones and co-chaperones, including p23 (Madon-Simon et al., 2017; Patwardhan et al., 2013) are directly involved in the regulation of apoptotic mechanisms in response to accumulation of misfolded proteins, oxidative stress, DNA damage, etc. As CyPA was shown to undergo subcellular relocalization upon the onset of stress (Fig. 3), comparative indirect immunofluorescence studies were also conducted for CyPA and various molecular chaperones. It was observed that CyPA shows significant colocalization with the Hsp90 co-chaperone p23. This was detected in all cell types, but it is particularly remarkable in neuronal cells. Therefore, the following studies were performed using the N2a neuroblastoma cell line. The potential interaction of CyPA with p23 is quite an interesting observation as CyPA is not an Hsp90 interactor (Galigniana et al., 2004). Fig. 5A shows that p23 undergoes the same subcellular relocalization as CyPA in N2a neuroblastoma cells exposed to H_2O_2. The scatter plot in Fig. 5B shows a two-dimensional histogram for both channels in which the intensity values of each pixel were plotted against each other and the overlapped colour profiles for CyPA and p23. Pixels possessing overlapping signals, as is the case with a true colocalization, plot along a 45° line, i.e. close to the pixels depicted here for the channels of p23 and CyPA (note the excellent linear regression coefficient r=0.956).

Because colocalization does not prove the existence of true heterocomplexes, p23 was immunoprecipitated from N2a cell extracts (Fig. 6A). As expected, Hsp90 and Hsp70 were co-immunoprecipitated, as p23 belongs to these type of complexes, and CyPA was also pulled down. To evaluate whether these complexes of p23 and CyPA require the presence of Hsp90, N2a cells were transfected with HA-tagged CyPA, and the IMM was pulled down with an anti-HA antibody (Fig. 6B). The co-chaperone p23 was recovered with CyPA in the immunopellet, but was free of Hsp90. These results indicate that CyPA and p23 are not associated via Hsp90, and that CyPA•p23 form in an unusual independent manner. Nevertheless, Hsp90 still forms separate complexes with p23 in these cells (as expected), as it was demonstrated by the co-immunoprecipitation of the chaperone with an anti-p23 antibody (Fig. 6A) that recognizes the co-chaperone in both types of complexes, Hsp90•p23 and CyPA•p23.

A piece of straightforward evidence that shows a direct association between CyPA and p23 is the far western blot shown in Fig. 6C. Immunopurified p23 was stripped, washed and resolved by 15% SDS-PAGE. The membrane was incubated with a dilution of purified recombinant GST-CyPA. The resultant complex of the IMM with the co-chaperone was visualized by enhanced chemiluminescence (ECL) using an anti-GST antibody and then a secondary counter-antibody tagged to horseradish peroxidase (HRP). The development of GST-CyPA on the band of p23 clearly indicates that the interaction of both proteins is direct. It should be remarked that PPIase enzymatic activity of CyPA does not appear to be required for its association with p23 as the blot showed identical results when it was performed in the presence of 1 µM cyclosporine A, a drug that inhibits the PPIase activity (data not shown).

Because the experiments shown in Fig. 4 suggested that Cyt c may form complexes with CyPA, we investigated whether this could be possible and whether p23 is also potentially a member of a ternary heterocomplex. Fig. 6D shows an immunoprecipitation of endogenous CyPA from untreated control N2a cells and N2a cells exposed to oxidative stress. The western blot confirmed that under basal conditions, CyPA and p23 are capable of interacting, but not Cyt c (which is entirely mitochondrial). However, the release of Cyt c favours the formation of ternary cytosolic complexes with CyPA and p23. Again, this may be the reason by which the nuclear accumulation of Cyt c may be impaired by CyPA upon the onset of oxidative stress (see Fig. 4C).

Overexpression of p23 enhances the anti-apoptotic effect of CyPA

With the purpose of investigating the influence of p23 overexpression on the cell response to a pro-apoptotic stimulus, N2a cells were transfected with the plasmid pRK5MCS-hp23, encoding for human p23, and stimulated with 20 µM H_2O_2 for 18 h. The percentage of cells showing fluorescence after an incubation with Annexin-V-FITC is shown in Fig. 7A. Clearly, p23 overexpression (see western blot) exerts a significantly protective action. As expected, such increased level of expression of the co-chaperone due to its transfection is

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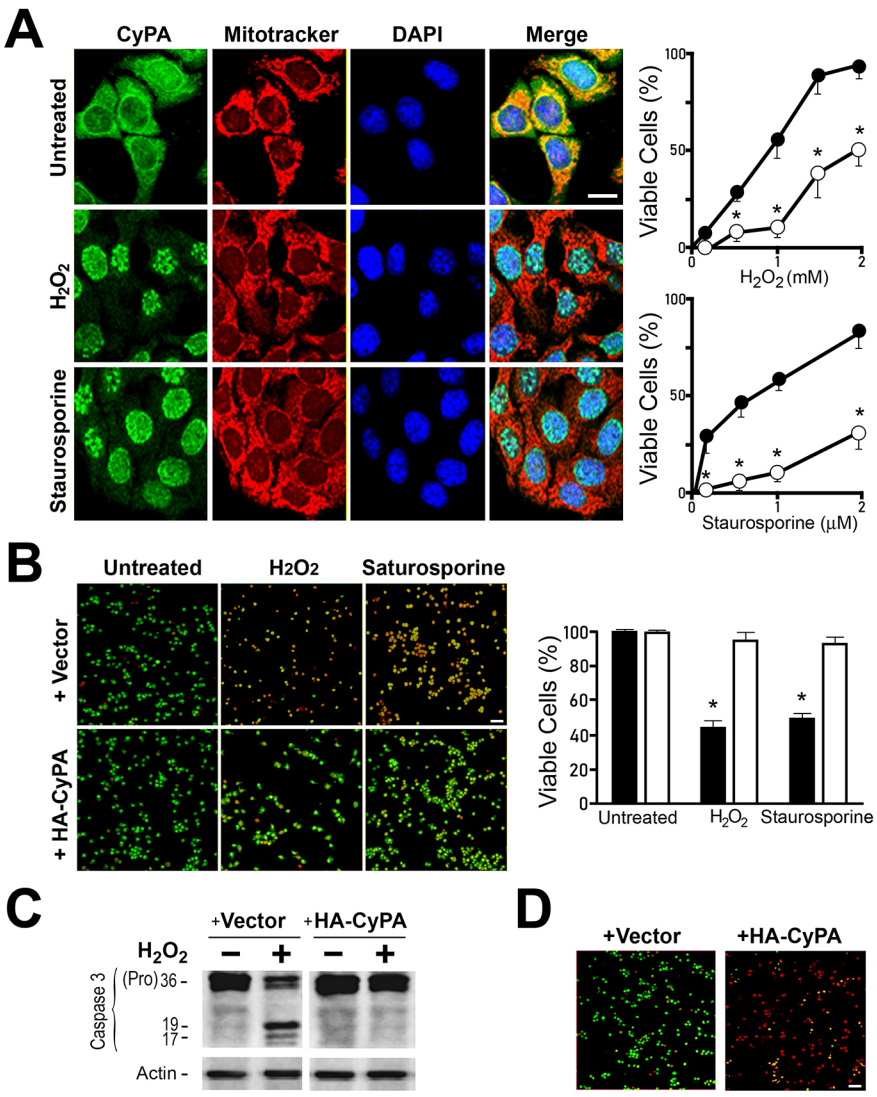

Fig. 3. CyPA shows anti-apoptotic properties. (A) Human 293T fibroblasts were treated for 4 h with 100 nM staurosporine or 0.25 mM H_2O_2, and fixed and stained with MitoTracker (red) or by indirect immunofluorescence for CyPA (green). Images obtained using confocal microscopy (Bar=10 µm). Cell viability plots (according to MTT assay) are shown on the right for cells transfected with pBEX1-HA-hCyPA (lines with solid circles) versus cells transfected with empty vector (lines with empty circles), and then exposed to the indicated concentrations of H_2O_2. The anti-apoptotic action of CyPA was also assayed in HA-CyPA 293T-overexpressing cells according to an acridine orange/ethidium bromide test after treatment with 100 nM staurosporine (B), procaspase 3 cleavage upon cell exposure to 0.25 mM H_2O_2 (C), and JC-1 cell staining after 10 µM etoposide stimulation (D). Data are mean±s.e.m. (n=6), *P<0.005 (Bonferroni test). Scale bars: 10 µm (A); 100 µm (B,D).

reflected in a higher recruitment of p23 to CyPA (see the inset showing the greater amount of co-immunoprecipitated p23 with the anti-CyPA antibody).

Next, the effect of the overexpression of both CyPA and p23 on the resistance to apoptosis was assayed. Fig. 7B demonstrates that a high level of expression of both proteins, CyPA and p23, in cells exposed to peroxide, greatly attenuated the cleavage of both procaspase 3 and vimentin, the proteolysis of which is considered a typical apoptotic response. In other words, the overexpression of both p23 and the IMM leads to the formation of larger amounts of CyPA•p23 complexes, enhancing the protective action of each individual protein in cells exposed to harmful conditions.

In the mitochondrial apoptotic pathway, Bcl2 plays a positive role in inhibiting cell death, whereas Bax is a pro-apoptotic factor (Cosentino and Garcia-Saez, 2017). In view of all these observations, it became mandatory to investigate whether CyPA and p23 exert any role in the expression levels of these two antagonistic proteins. Fig. 7C demonstrates that the overexpression of any individual protein induced the expression of Bcl2 and decreases Bax levels, an effect that was potentiated by the double overexpression of CyPA and p23. This observation clearly demonstrates the direct influence of both proteins of the novel heterocomplex to favour cell survival.

Role of extracellular CyPA

It is well known that CyPA is secreted and exerts prosurvival actions in neurons by binding to the CD147 plasma membrane receptor (Boulos et al., 2007; Yurchenko et al., 2002). Actually, CyPA can be secreted from several cell types in response to oxidative stress, hypoxia, infection and various inflammatory stimuli (Nigro et al., 2013). Because CyPA secretion is greater when its expression is high, and because in our hands the overexpression of CyPA increased cell protection against pro-apoptotic stimuli, we wondered whether these anti-apoptotic effects may also be related to an extracellular mechanism. Fig. 8 shows that N2a cell viability was favoured when these cells were incubated with purified recombinant CyPA (bar 3 versus bar 2), and also when the conditioned medium of CyPA-overexpressing cells was offered to normal cells (bar 4 versus bar 2). However, no differences were observed when the conditioned medium was obtained from p23-overexpressing cells (bar 5). The western blot under the plot shows the expression of Bcl2 in these cells for each condition. In agreement with the observed effect on cell survival, the expression of Bcl2 increased in cells stimulated by both types of extracellular CyPA.

DISCUSSION

In this study, it was demonstrated that CyPA is a mitochondrial factor that migrates to the nucleus upon the onset of harmful stimuli, such as

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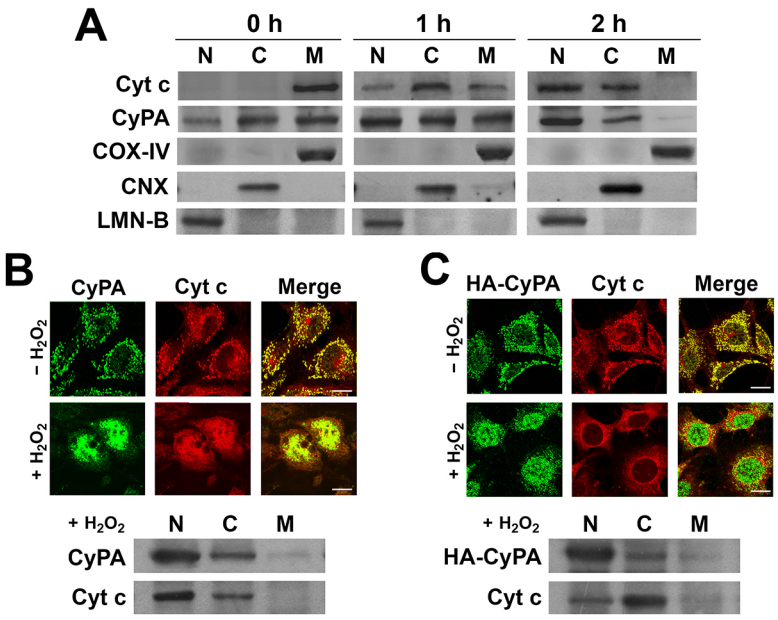

Fig. 4. CyPA and Cyt c release from mitochondria. (A) HeLa cells were treated with 0.25 mM H_2O_2 for 1 h and 2 h. After a biochemical fractionation, proteins from the nuclear (N), cytosolic (C) and mitochondrial (M) fractions were resolved by western blot for Cyt c, CyPA, and the protein markers COX-IV for mitochondria, CNX for endoplasmic reticulum and vesicle-like artifacts, and lamin B (LMN-B) for nuclei. (B) Indirect immunofluorescence for endogenous Cyt c (red) and endogenous CyPA (green) in HeLa cells exposed or not exposed to 0.25 mM H_2O_2 for 4 h. (C) Indirect immunofluorescence for endogenous Cyt c (red) and exogenous HA-CyPA (green staining for anti-HA antibody) in HeLa cells exposed or not exposed to 0.25 mM H_2O_2 for 4 h. Western blots below B and C show the subcellular distribution of both proteins in cells treated with H_2O_2. HA-CyPA was developed with the anti-HA antibody. Scale bars: 10 µm.

oxidative stress and exposure to staurosporine or etoposide. Also, CyPA showed anti-apoptotic properties and the ability to form heterocomplexes with the co-chaperone p23 and Cyt c. Interestingly, the high levels of expression of the IMM and the co-chaperone reached by transfection, but physiologically by endogenous induction in stressed or cancer cells, favours the formation of CyPA•p23 complexes,

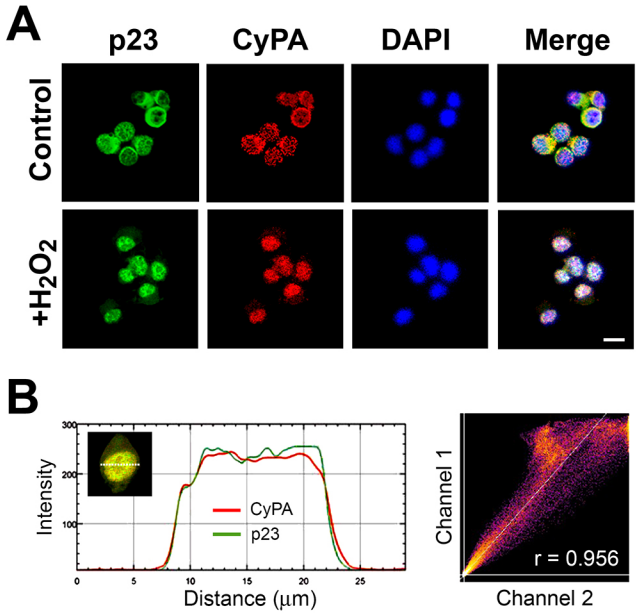

Fig. 5. CyPA colocalizes with the co-chaperone p23. (A) Indirect immunofluorescence for endogenous CyPA (red) and p23 (green) in N2a neuroblastoma cells. Both proteins co-migrate to the nucleus in the presence of 20 µM H_2O_2. Scale bar: 10 µm. (B) The overlapping colour profile for CyPA (red) and p23 (green). The cytofluorogram depicts the correlation between the intensities of the two channels of the pixels over the distance (in µm) along the nucleus shown in the inset (white-dotted line). The two-dimensional histogram depicts the intensity values for each voxel when both dyes are plotted against each other in a *z*-stack of the nucleus. The brighter the colour the more voxels have those two intensity values for their two channels. r, linear regression coefficient.

and the anti-apoptotic effect shown by each individual protein is enhanced. It is important to note that the stimuli do not generate cell death by autophagy or necrosis, but apoptosis, as it is shown by the different assays, demonstrated this mechanism of cell death (i.e. procaspase and vimentin cleavage, annexin-V binding to plasma membrane, changes in the mitochondrial potential evidenced with JC-1, Bcl2/Bax expression ratio, etc.). Remarkably, the subcellular localization of CyPA, as well as its nuclear translocation triggered by stress, was a phenomenon evidenced in all cell types assayed by both confocal microscopy studies and standard biochemical fractionations. This suggests that this property is not restricted to a specific cell type. Furthermore, the mitochondrial localization of CyPA was also evidenced in rat liver mitochondria (Fig. 2) and other organs, such as the brain and spinal cord (data not shown).

Interestingly, our study indicates that the novel association of CyPA with the small acidic co-chaperone p23 does not require Hsp90, a chaperone that itself usually requires p23 to stabilize its heterocomplexes (Forafonov et al., 2008; Freeman et al., 1996; Pratt et al., 2004b). Importantly, p23 has also been indirectly associated with anti-apoptotic actions in other studies. Thus, an exacerbated instability of p23 leads to breast and cervical carcinoma cells, and apoptosis-mediated death (Patwardhan et al., 2013), suggesting a protective role for p23. Accordingly, cancer and metastatic cells show higher levels of p23 expression (Mollerup et al., 2003; Oxelmark et al., 2006); the prognosis is worse in prostate cancer patients with increased nuclear localization of p23 (Cano et al., 2015), and its overexpression protects cells against the deleterious actions of Hsp90 inhibitors (Forafonov et al., 2008). A similar situation is caused by CyPA upregulation in malignant transformation and several types of cancers (Lee, 2010). Therefore, the potentiation of CyPA•p23 as a novel anti-apoptotic complex may be linked to improved survival skills of cancer cells, such that the disruption of this association by drugs deserves to be attempted as a novel putative target against cancer.

Moreover, CyPA was also capable of interacting with Cyt c, an observation that confirmed that reported by Bonfils et al. (2010). In our study, it is also demonstrated that the novel heterocomplex CyPA•p23 sequesters Cyt c within the cytoplasm, a fact that may be related to the prevention of the activation of the mitochondrial pro-apoptotic pathway. Interestingly, the data presented in Fig. 4

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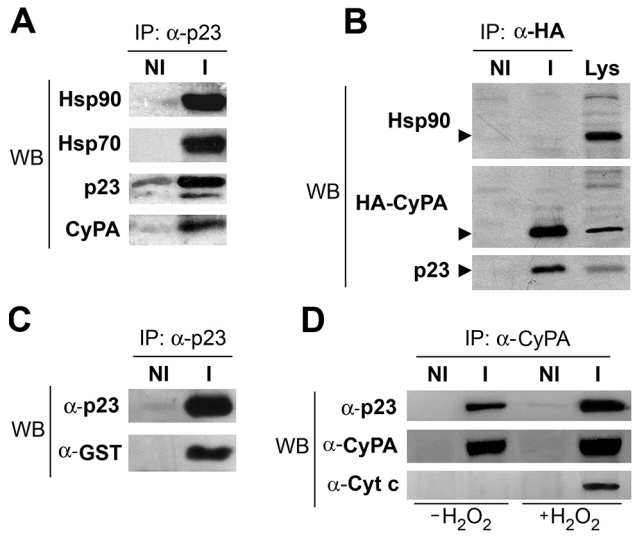

Fig. 6. CyPA and p23 interact directly. (A) N2a cytosol was immunoprecipitated (IP) with an anti-p23 IgG antibody (I). A mouse non-immune IgG antibody (NI) was used as a control. Proteins were resolved in a 15% SDS-PAGE gel, followed by western blot (WB) for the indicated proteins. (B) N2a cells transfected with pBEX1-HA-hCyPA were homogenized. The lysate (Lys) was immunoprecipitated with an anti-HA IgG antibody (I) or a non-immune mouse IgG antibody (NI), and proteins were resolved by western blotting as in panel A, except that CyPA was revealed with anti-HA IgG antibody. (C) The co-chaperone p23 was immunoprecipitated with an anti-p23 IgG antibody, stripped of associated proteins and resolved by SDS-PAGE. The membrane was incubated with a solution of purified GST-CyPA and the potential interaction of the IMM with p23 was examined by western blotting with an anti-GST antibody and an HRP-tagged counter antibody. Then, the membrane was stripped and western blotted for p23. (D) Endogenous CyPA was immunoprecipitated after N2a cells were exposed to 20 µM H_2O_2 for 1 h. The endogenous tripartite heterocomplex CyPA•Cyt c•p23 generated by exposure of cells to peroxide was resolved by western blotting. The peroxide-dependent recruitment of Cyt c to the CyPA•p23 cytosolic complex can also be observed.

demonstrate that the mitochondrial export rate of both CyPA and Cyt c is similar, and the higher expression levels of CyPA makes Cyt c mostly cytoplasmic (rather than nuclear), presumably because it is associated with the tripartite anti-apoptotic heterocomplex CyPA•Cyt c•p23. It has been demonstrated that the nuclear accumulation of Cyt c during the onset of pro-apoptotic stimuli induces the nuclear release of acetylated histones and chromatin condensation (Nur et al., 2004), two events that are directly involved in the remodelling of chromatin during the response of the cell to adverse situations.

It is interesting to note that another IMM that belongs to the FK506-binding protein subfamily, FKBP51, is also a mitochondrial protein that migrates to the nucleus upon the onset of stress, and also shows anti-apoptotic behaviours (Gallo et al., 2011). Like CyPA, FKBP51 is also highly expressed in several types of cancer cells (Lagadari et al., 2016), and is able to interact with NF-κB (Erlejman et al., 2014). CypA also interacts with the N-terminal region of p65/RelA (Sun et al., 2014), as well as human PIN1 (Ryo et al., 2003). Despite having different substrate recognition motifs, similar biological functions have been observed for various PPIases, which show some features in common that allow them to occasionally complement one another. As with FKBP51 and Pin1, the upregulation of CypA in most tumours is associated with tumour aggressiveness, an effect that could also be complementary to the anti-apoptotic action. This makes all of these PPIases good candidates for potential anti-cancer therapies using small molecule inhibitors.

In addition to its classic role in protein folding, CyPA has also been involved in intracellular trafficking of soluble proteins (Galigniana et al., 2004; Zhu et al., 2007), signal transduction (Sun et al., 2014) and transcriptional regulation (Xie et al., 2019). Other members of the cyclophilin subfamily were cloned and characterized after CyPA identification, among them CyPD, which shows 75% structural homology with CyPA (Mehta, 2018), and is an IMM linked to the inner mitochondrial membrane in the mitochondrial permeability transition pore. In contrast to CyPA oligomeric properties, CyPD is an Hsp90-associated partner and requires this chaperone (Kang et al., 2007). Although it binds cyclosporine A, its biological functions are independent of the PPIase activity (Scorrano et al., 1997). In contrast to the observed effects of CyPA overexpression, increased levels of CyPD, like those shown in most neurodegenerative diseases, leads to cell death due to colloidal osmotic swelling of the mitochondrial

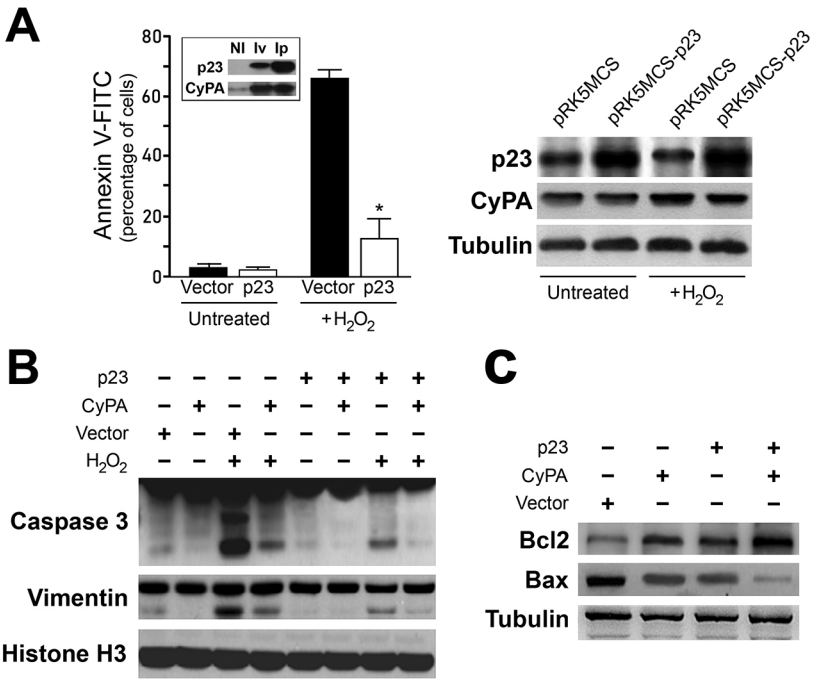

Fig. 7. CyPA and p23 overexpression favours an anti-apoptotic effect. (A) N2a cells transfected with pRK5MCS-hp23 were exposed to H_2O_2, and the percentage of Annexin-V^+ cells was quantified by flow cytometry. Data are mean± s.e.m. of three independent experiments, each one quantified in triplicate. *P<0.001 (significantly different form cells transfected with empty vector and treated with peroxide, Newman–Keuls test). The western blot on the right shows the protein profile for each type of lysate, and the inset shows the greater specific recruitment of p23 to the CyPA immunopellet in p23-transfected cells (Ip) versus the immunopellet obtained from extracts of cells transfected with empty vector (Iv). (B) Cells were co-transfected with both proteins, CyPA and p23, and the cleavage of procaspase 3 and the intermediate filament vimentin were evaluated by western blotting after the treatment with H_2O_2. (C) The Bcl2/Bax expression ratio improves in N2a cells overexpressing CyPA or p23, and is enhanced by the transfection of these two proteins.

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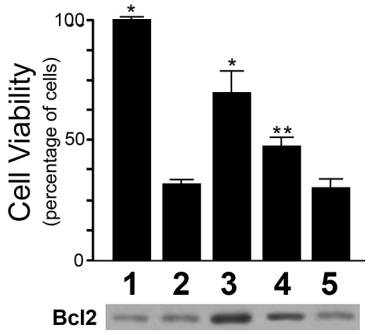

Fig. 8. Extracellular CyPA also shows anti-apoptotic action. N2a cells were exposed to 20 µM H_2O_2 for 12 h, and cell viability was evaluated by MTT test. The induction of the Bcl2 expression was evidenced by western blotting. Conditions were: (1) unstimulated control cells; (2) cells treated with peroxide for 12 h; (3) cells pre-incubated for 4 h with purified recombinant GST-CyPA (1 µg/ml), and then stimulated for 12 h with peroxide (CyPA being present during the incubation); (4) regular N2a cells were pre-incubated for 4 h with conditioned medium aspired from a culture of N2a cells transfected 24 h before with pBEX1-HA-hCyPA. Peroxide was added to the conditioned medium and the incubation continued for 12 h; and (5) the same experiment as in condition 4, but the conditioned medium was from a culture of N2a cells transfected 24 h before with pRK5MCS-hp23. The western blot at the bottom shows the expression of Bcl2 for each condition. Data are mean±s.e.m. (n=5). *P<0.001, **P<0.010 (significantly different from condition 2, Newman–Keuls test).

matrix, dissipation of the inner membrane potential, generation of reactive oxygen species, and the release of many pro-apoptotic proteins and procaspases (Zhang et al., 2015). Importantly, in contrast to mitochondrial CyPD, which is a protein associated with membranes that have a role in the regulation of calcium pores, mitochondrial CyPA is not confined to the organelle, and can fully accumulate in the nucleus, as demonstrated in this study. In other words, CyPA and CyPD do not exhibit redundant functions regardless of their high homology.

As with most members of the subfamily, the stress-inducible properties of CyPA are likely protective against the deleterious side effects of the stress response, and could be an extension of its intrinsic molecular chaperoning nature under normal conditions, a property that is especially exacerbated during harmful circumstances. These features are perhaps the main biological reason to favour associations of CyPA with key members of the chaperone family, such as the Hsp90 co-chaperone p23. Moreover, the beneficial association of CyPA and p23 may be a cardinal requirement for their involvement in the structural transformation that ultimately leads to mitochondria stabilization and the success of the anti-apoptotic effect.

MATERIALS AND METHODS

Reagents, cells and plasmids

Staurosporine, proteinase K, MTT, paraformaldehyde, and hydrogen peroxide (S180505-R22-4) were obtained from Sigma-Aldrich. Acetone was from Merck. Vectashield antifade mounting medium for microscopy was purchased from Vector Laboratories. All cell types used in this study were purchased from ATCC. Dulbecco's modified Eagle's medium (DMEM) and Opti-MEM culture media were obtained from Invitrogen. All plasmids encoding for human CyPA (pGEX-2T-GST-CyPA, pBEX1-HA-hCyPA and the inactive mutant pBEX1-HA-hCyPAH126Q) were a generous gift from Dr Philippe A. Gallay (Scripps Research Institute, La Jolla, CA, USA). The plasmid pRK5MCS-hp23, encoding for human p23, was kindly provided by Dr Theo Rein (Max Planck Institut für Psychiatrie, Munich, Germany). All cell lines used in this study are from ATCC, validated and routinely tested for contaminations in our institutional cell culture core.

Antibodies

The AC88 mouse monoclonal IgG antibody against Hsp90 (SPA-830) and the N27F3-4 anti-72/73-kDa heat-shock protein monoclonal IgG (anti-Hsp70) (SPA-820) were obtained from StressGen. The JJ3 mouse monoclonal IgG antibody against p23 (MA3-414) was obtained from Affinity BioReagents. The 6E2 mouse IgG anti-hemagglutinin (HA)-tag (H1847-53A) was obtained from US Biological. The rabbit IgG anti-caspase 3 antibody (9664L), the 124 mouse IgG anti-Bcl2 antibody (15071) and the D2E11 rabbit IgG antibody against Bax (5023S) were purchased from Cell Signaling Technology. The anti-CyPA goat polyclonal antibody (PA5-18463) and commercial rabbit antiserum (PA1-025) were purchased from Invitrogen. The rabbit polyclonal IgG anti-CyPA antibody (GTX104698) was obtained from GeneTex. Rabbit antiserum anti-CyPD antibody was a kind gift from Dr. Yolanda Sopena (University of Buenos Aires, Argentina). The TSRI polyclonal IgG antibody against CyPA was prepared by Dr Philippe Gallay at TSRI Antibody Core Facility by immunization of rabbits with recombinant human CyPA protein (Saphire et al., 1999). Anti-GST rabbit antiserum was a generous gift from Dr Martín Monte (University of Buenos Aires, Argentina). Mouse antibodies against the cytoskeletal proteins vimentin (AB4701011), actin (A3853) and β-tubulin (T5211) were obtained from Sigma-Aldrich. The mitochondrial marker MitoTracker, JC-1 dye, annexin-FITC and all fluorescent secondary antibodies were obtained from Molecular Probes. Rabbit monoclonal IgG anti-histone H3 antibody (05-499) was purchased from Upstate Biotechnology. Goat polyclonal IgG anti-lamin B antibody (sc-6216), mouse monoclonal IgG antibodies against cytochrome c (sc-13156), anti-Tom-20 antibody (sc-17764), goat polyclonal IgG anti-lamin B antibody (sc-6216) anti-NuMa antibody (sc-18555) and mouse monoclonal IgG anti-calnexin antibody (sc-46669) were obtained from Santa Cruz Biotechnology. Anti-COX-IV antibody (ab14744) was purchased from Abcam. Immunoprecipitation assays were performed as described previously (Mazaira et al., 2020; Piwien-Pilipuk et al., 2002).

Microscopy studies

Cells were grown on coverslips and processed for indirect immunofluorescence, as described in previous studies (Presman et al., 2006; Salatino et al., 2006; Toneatto et al., 2013). Briefly, the cells were fixed at room temperature for 1 h in a freshly prepared solution of 4% paraformaldehyde in PBS, and then permeabilized for 5 min in ice-cold (−20°C) acetone. After being washed with PBS buffer, the coverslips were pre-incubated for 1 h at room temperature in high ionic strength solution (HISS) buffer (20 mM Tris at pH 8.0, 0.63M NaCl, 0.1% Tween 20 and 3.5% bovine serum albumin) supplemented with 1% horse serum, and then incubated for 2 h at 4°C with a 1/250 dilution (in HISS buffer) of primary antibody, followed by a 1-h incubation at room temperature with 1/200 dilution of secondary antibody. The coverslips were mounted in a glycerol-based medium with an antifade solution. Confocal microscope images were acquired using a Nikon Eclipse-E800 confocal microscope, with a Nikon DSU1 camera, and ACT-2U software (v.1.70) from Nikon Tec Corp. Colocalization analyses were performed using the colocalization plug-in of Fiji (v.1.45, National Institutes of Health), which uses a range of algorithms, such as colocalization thresholds, Pearson's linear correlation coefficient, overlap and Manders' coefficients (Manders et al., 1993). We collected confocal z-series of cells (40 optical slices, airy unit ¼ 1 airydisc at 0.25 mm intervals using a 63× objective). Images were then deconvolved using Huygens compute engine 3.5.2p3 64b (closed platform). Finally, images were imported to Fiji to determine the degree of overlapping.

Cell fractionation and proteinase K digestion

Mitochondria were isolated from rat liver or cell extracts following a standard procedure for biochemical fractionation described previously (Gallo et al., 2011). Briefly, cell extracts were made in buffer H (20 mM HEPES at pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, 250 mM sucrose and a protease inhibitors cocktail) and centrifuged at 4°C for 5 min (800 ***g***), yielding a supernatant (S1a) and a pellet (P1). Because this pellet entraps mitochondria, it was subjected to a second round of centrifugation under the same conditions in a 50 ml centrifuge tube with buffer H. This supernatant (S1b) was carefully removed

and mixed with S1a. The pellet was resuspended in buffer B (20 mM Tris pH 7.5, 1.5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM EGTA, 70 mM sucrose, 210 mM mannitol and a protease inhibitors cocktail). The sample was gently placed on a dense layer of 2.1 M sucrose and subjected to high speed centrifugation (65,000 ***g*** for 30 min at 4°C in a Beckman L7 ultracentrifuge using an SW28 rotor) to separate according to their density, as well as the nuclei from contaminant mitochondria and membranes. The nuclear pellet was washed four times with buffer B by centrifugation at 1500 ***g*** over a period of 20 min. This step also removes many tissue remnants when isolating nuclei from tissues (Nabbi and Riabowol, 2015). To isolate mitochondria, both S1 supernatants were centrifuged at 10,000 ***g*** for 20 min at 4°C, yielding a crude mitochondrial pellet, P2. The supernatant S2 was centrifuged in the Beckman L7 centrifuge for 45 min at 80,000 ***g*** using a 50Ti rotor. The pellet was composed of microsomes and vesicle-like artifacts, and the resultant supernatant was used as the 'cytosolic' fraction. The P2 pellet was washed four times in buffer B and centrifuged at 10,000 ***g*** for 10 min at 4°C. The washed pellet was resuspended in 10 volumes with the same buffer and divided into 1.5-ml Eppendorf tubes containing 100 µl of 50% sucrose at the bottom of the tube and 100 µl of 40% sucrose on top of the previous layer (Ansari et al., 2015; Graham, 1993). The tubes were centrifuged at 24,500 ***g*** for 3 min in a microfuge placed in the cold room. Under these conditions, almost all peroxisomes are pooled on the bottom of the tube (as judged by the measurement of catalase activity), and most lysosomes are trapped in the middle layer (as judged by the acid phosphatase enzymatic activity). The mitochondrial interfaces are recovered on the shallow layer. Pools of two Eppendorf tubes were transferred to a single tube, and the previous procedure was repeated, such that the pellets from two tubes were combined to obtain a single pellet originating from four Eppendorf tubes. The procedure was repeated, and the final mitochondrial pools were washed by resuspension in 1 ml of buffer B, and centrifuged. The pellets were used immediately or stored at −80°C. Markers for the purification procedure for each subcellular fraction were lamin B and NuMa for nuclei, calnexin for microsomes and vesicle-like contaminants, COX-IV and cytochrome c for mitochondria, and the enzymatic activities of acid phosphatase and catalase for lysosomes and peroxisomes, respectively (Fernandez-Vizarra et al., 2002). Mitochondrial pellets were resuspended in a minimal volume of 10 mM HEPES buffer at pH 7.4 supplemented with 1% SDS and protease inhibitor cocktail. Protein concentration was measured and standardized using a NanoDrop spectrophotometer. Proteins were resolved by western blot, and developed by ECL. In all western blots, aliquots of whole-cell lysates (referred in the figures as 'Lysate') were also developed as expression markers of the investigated protein.

In order to determine whether CyPA is intramitochondrial rather than associated with the outer membrane of the organelle, isolated mitochondria were permeabilized by pretreatment with 1% SDS for 10 min at 4°C, and then incubated for an additional 15 min with either 5 or 50 ng/ml proteinase K (Kang et al., 2007). The presence of CyPA was resolved by western blot in a 15% SDS-PAGE gel.

Far western blot

Proteins from 3T3-L1 fibroblast lysates were extracted with Cytobuster protein extraction reagent (Novagen, Merck). After cell disruption for 10 min at room temperature, the extracts were centrifuged at 10,000 ***g*** for 10 min. The co-chaperone p23 was immunoprecipitated with JJ3 anti-p23 mouse monoclonal IgG antibody, washed three times with TEGMo buffer (10 mM TES at pH 7.6, 50 mM NaCl, 4 mM EDTA, 10% v/v glycerol and 20 mM Na_2MoO_4), and proteins were resolved by a 15% SDS-PAGE gel, transferred to an Immobilon-P membrane and incubated with a recombinant GST-CyPA solution obtained from IPTG-induced *Escherichia coli* as described previously (Galigniana et al., 2004, 2001). After washing the membrane, the attachment of GST-CyPA to p23 was visualized by ECL with anti-GST antiserum/donkey anti-rabbit IgG-HRP antibody.

Apoptosis and cell viability tests

Cell viability was estimated according to the MTT assay (Vibrant Assay Kit, Thermo Fisher Scientific). Procaspase and vimentin cleavage was assayed as described previously (Gallo et al., 2011). An acridine orange/ethidium bromide test was performed as described previously (Liu et al., 2015). Cell viability was assayed as reported previously (Colo et al., 2008; Lagadari et al., 2016). Etoposide-induced apoptosis in human 293T fibroblasts was induced as described previously (Attardi et al., 2004). Cell staining with JC-1, annexin-FITC and MitoTracker markers was accomplished as described previously (Gallo et al., 2011). To evaluate the effect of extracellular CyPA on cell viability, N2a cells were pre-incubated with purified recombinant GST-CyPA (1 µg/ml) for 4 h, and then cells were exposed to 20 µM H_2O_2 for 12 h. Alternatively, N2a cells were grown on conditioned medium for CyPA overexpressing N2a cells, and then they were exposed to H_2O_2. An MTT viability assay was performed and the induction of endogenous Bcl2 was evaluated by western blotting.

Statistical analysis

Data are mean±s.e.m. Normality of variables was explored using the Shapiro–Wilk test, and homogeneous variances using the Levene test. The statistical comparisons of parametric variables for more than two groups were performed using one-way ANOVA followed by the Newman–Keuls post-hoc test, and for two categories of variables the two-way ANOVA was used, followed by the Bonferroni post-hoc test.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.L., M.D.G.; Methodology: C.D.-B., B.V., L.I.G.; Software: C.D.-B., B.V., M.L.; Validation: C.D.-B., B.V., L.I.G.; Formal analysis: C.D.-B., B.V., M.L., M.D.G.; Investigation: C.D.-B., B.V., L.I.G.; Resources: M.D.G.; Data curation: C.D.-B., B.V., L.I.G., M.L.; Writing - original draft: M.L., M.D.G.; Writing - review & editing: M.D.G.; Visualization: M.L.; Supervision: M.L., M.D.G.; Project administration: M.D.G.; Funding acquisition: M.D.G.

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Peer review history

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