



# Oxidative stress is tightly regulated by cytochrome *c* phosphorylation and respirasome factors in mitochondria

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Edited by Alan R. Fersht, Gonville and Caius College, Cambridge, United Kingdom, and approved June 26, 2018 (received for review April 20, 2018)

Respiratory cytochrome *c* has been found to be phosphorylated at tyrosine 97 in the postschismic brain upon neuroprotective insulin treatment, but how such posttranslational modification affects mitochondrial metabolism is unclear. Here, we report the structural features and functional behavior of a phosphomimetic cytochrome *c* mutant, which was generated by site-specific incorporation at position 97 of *p*-carboxymethyl-L-phenylalanine using the evolved tRNA synthetase method. We found that the point mutation does not alter the overall folding and heme environment of cytochrome *c*, but significantly affects the entire oxidative phosphorylation process. In fact, the electron donation rate of the mutant heme protein to cytochrome *c* oxidase, or complex IV, within respiratory supercomplexes was higher than that of the wild-type species, in agreement with the observed decrease in reactive oxygen species production. Direct contact of cytochrome *c* with the respiratory supercomplex factor HIGD1A (hypoxia-inducible domain family member 1A) is reported here, with the mutant heme protein exhibiting a lower affinity than the wild-type species. Interestingly, phosphomimetic cytochrome *c* also exhibited a lower caspase-3 activation activity. Altogether, these findings yield a better understanding of the molecular basis for mitochondrial metabolism in acute diseases, such as brain ischemia, and thus could allow the use of phosphomimetic cytochrome *c* as a neuroprotector with therapeutic applications.

cytochrome *c* | electron transport chain | oxidative stress | phosphorylation | reactive oxygen species

Cell redox signaling is fine-tuned by protein phosphorylation, a well-known posttranslational modification in turn modulated by the antagonistic effects of kinases and phosphatases (1, 2). Such an interplay is specifically relevant in mitochondrial metabolism, which is the main source of reactive oxygen species/reactive nitrogen species (ROS/RNS) in the cell (3). Tyrosine kinases, in particular, are key signaling mediators and their perturbations underlie many human pathologies, namely cancer and neurodegenerative diseases (4, 5). One of the main proteins that control redox signaling in mitochondrial oxidative phosphorylation (OxPhos) is cytochrome *c* (*Cc*), the functions of which are regulated by phosphorylation of several residues (6–10).

*Cc* plays a double role in the cellular metabolism. Under homeostasis, *Cc* is located in the mitochondrial intermembrane space, where it acts as an electron carrier between complex III (CIII) and complex IV (CIV) in the electron transfer chain (ETC). These two respiratory complexes can be either independent or associated with each other and with complex I (CI), thus forming mitochondrial supercomplexes—the so-called respirasome—the assembly of which is strictly dependent on metabolic needs (11). Respiratory supercomplexes provide higher efficiency to the electron transport during OxPhos, thereby minimizing the generation of ROS (12). Respiratory supercomplex factors (Rcfs) are protein modulators that stabilize the membrane-embedded CIII and CIV within respiratory

supercomplexes. These factors have been found in both yeasts and mammals. In yeasts, they are called respiratory supercomplex factor 1 (Rcf1, formerly Aim31) and respiratory supercomplex factor 2 (Rcf2, formerly Aim38), both being members of the conserved hypoxia-induced gene 1 (Hig1) protein family (13, 14). Rcf1 has two human orthologs, namely the HIG hypoxia-inducible domain family members 1A and 2A (HIGD1A and HIGD2A, respectively) (15). Rcf2, in contrast, is yeast-specific and necessary for oligomerization of a subclass of *Cc* oxidase (*Cc*O) into supercomplexes.

Under nitro-oxidative stress conditions, *Cc* acts as a programmed cell death (PCD) inducer (16). During early apoptosis, a *Cc* population—tightly bound to the mitochondrial membrane—triggers peroxidation of phospholipids and, in particular, of cardiolipin (CL). CL-adducted *Cc* undergoes a profound tertiary conformational rearrangement that opens an entry channel for H<sub>2</sub>O<sub>2</sub> molecules, which explains how *Cc* enhances its peroxidase activity (17, 18). It has been proposed that *Cc*-CL conjugates are sufficient for formation of mitochondrial pores, allowing the release of the heme protein into the cytosol during apoptosis (19).

Extramitochondrial *Cc* binds to the apoptotic protease-activating factor 1 (Apaf1), thereby eliciting the apoptosome

## Significance

Dysfunction of mitochondria, the powerhouses of living cells, favors the onset of human diseases, namely neurodegenerative diseases, cardiovascular pathologies, and cancer. Actually, respiratory cytochrome *c* has been found to be phosphorylated at tyrosine 97 during the insulin-induced neuroprotection response following a brain ischemic injury. Here, we report that the decrease in neuronal death could be directly ascribed to changes in mitochondrial metabolism—including lower production of reactive oxygen species—and cell homeostasis induced by cytochrome *c* phosphorylation. Our findings thus provide the basis for understanding the molecular mechanism and potential use of phosphomimetic species of cytochrome *c*, thereby yielding new opportunities to develop more efficient therapies against acute pathologies.

Author contributions: A.G.-C., A.D.-Q., M.A.R., and I.D.-M. designed research; A.G.-C., G.P.-M., C.A.E.-R., K.G.-A., S.M.G.-M., and I.D.-M. performed research; A.G.-C., A.D.-Q., G.P.-M., C.A.E.-R., and I.D.-M. analyzed data; and A.G.-C., A.D.-Q., M.A.R., and I.D.-M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1806833115/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1806833115/-DCSupplemental).

Published online July 17, 2018.

**Table 1. Midpoint melting temperature ( $T_m$ ) (°C) of WT and Y97pCMF Cc**

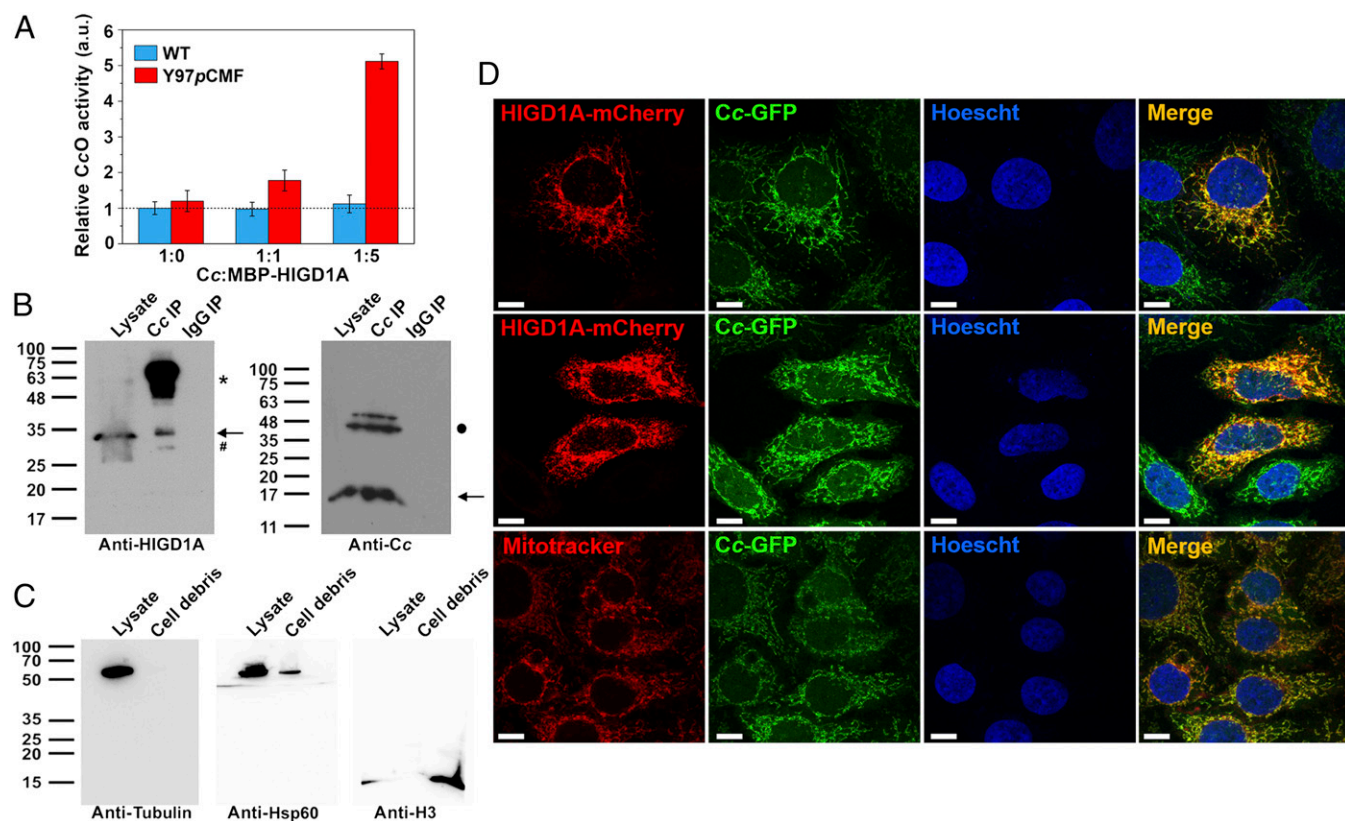
| Cc species | Far-UV<br>CD,<br>220 nm | Visible CD,<br>419 nm                              | Fluorescence,<br>270 nm |
|------------|-------------------------|--|-------------------------|
| WT         | 88.6 ± 1.9              | 43.9 ± 2.0 ( $T_{m1}$ )<br>85.1 ± 2.3 ( $T_{m2}$ ) | 86.3 ± 1.4              |
| Y97pCMF    | 82.2 ± 0.3              | 76.6 ± 4.9   | 82.6 ± 1.6              |

platform assembly and activating the caspase cascade (20, 21). Recent data indicate that the programmed cell death network involving Cc is complex, and that Cc targets several proteins that are functionally equivalent in humans and plants (22–24). In both type of cells, for example, Cc reaches the cell nucleus upon DNA damage and sequesters human SET/TAF1 $\beta$  and plant NRP1 histone chaperones that remodel chromatin (25–27).

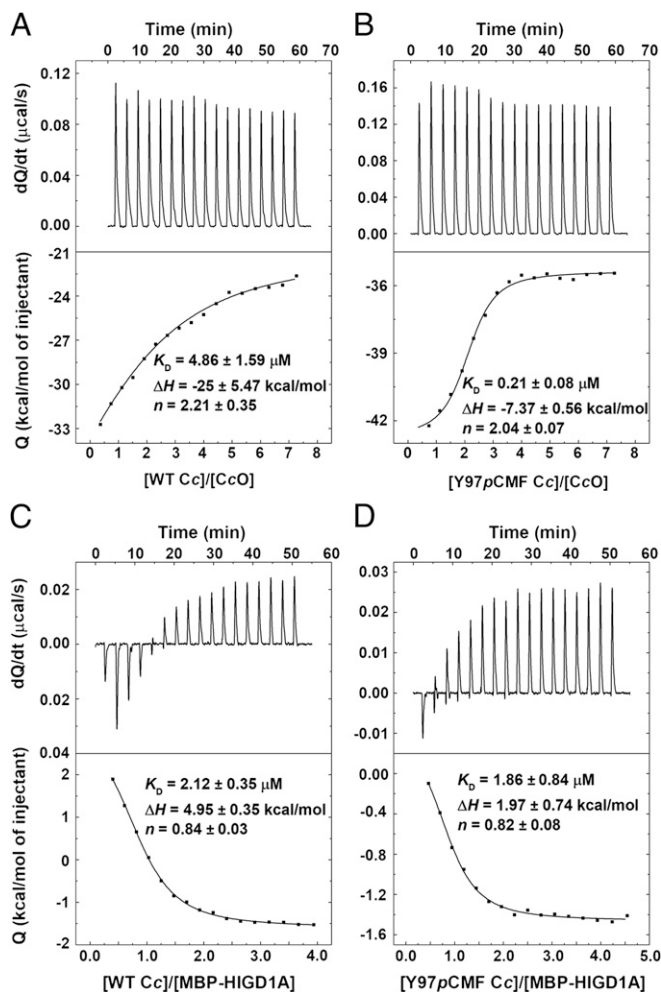
In addition to regulating the functions described above, phosphorylated Cc species have been associated with several human pathologies. In fact, it has been proposed that Tyr97 is target of phosphorylation during the insulin-induced neuroprotection response following a brain ischemic injury (28). A possible mechanism could be that Tyr97 phosphorylation of Cc would alter the electron shuttling to CcO (7). Notably, electron

transfer from wild-type (WT) Cc to CcO is positively regulated by the HIGD1A membrane protein (29).

Research on tyrosine phosphorylation of Cc has normally resorted to using Tyr-to-Glu mutations (30, 31). This overcomes the difficulties of obtaining enough phosphorylated Cc for biophysical and functional studies when the specific Cc-phosphorylating kinase is unknown (32). However, the Tyr-to-Glu substitution leads to a substantial decrease in the residue volume and the loss of an aromatic ring. Therefore, we used the evolved tRNA synthetase technique to introduce the non-canonical amino acid *p*-carboxymethyl-L-phenylalanine (*p*CMF) at position 97, as previously reported for Tyr48 (33). This non-canonical amino acid is actually used to synthesize stable analogs of a wide array of phosphoproteins, the kinases of which are unknown or the yields of which from tissues are low. In addition, *p*CMF provides a dependable analog of phosphorylated tyrosine that can be used to study signal transduction pathways. In this work, the resulting Y97*p*CMF Cc mutant maintained its overall folding and heme environment. We found that such a post-translational modification of Cc affected the OxPhos process since it enhanced the electron donation rate to CcO in mitochondria isolated from yeasts grown in respiratory metabolism. In addition, we observed a decrease in the production of ROS. Thus, the CcO-driven oxidation rate of Cc is controlled by



**Fig. 1.** Modulation of CcO activity by the HIGD1A protein and interaction of the latter with Cc. (A) Bovine CcO activity with added WT (blue) or Y97*p*CMF (red) Cc as electron donor in the presence of the chimera MBP-HIGD1A at varying concentrations. The 1:0 bars represent the CcO activity without MBP-HIGD1A. Experimental data were normalized to the Cc:HIGD1A ratio of 1:0 for WT Cc species. a.u.: arbitrary units. (B) Immunoprecipitation of transfected HIGD1A-mCherry with Cc in Heltog cells. (Left) Western blot shows the detection of the HIGD1A-mCherry band in the lysates, along with Cc-IP of lysates followed by probing with the HIGD1A antibody (arrow). Mouse IgG (IgG) was used as control. (Right) Detection of Cc band in lysates and Cc-immunoprecipitated lysates are also shown (arrow). Asterisks and hashtags are heavy and light chains of IgG, respectively. The black circle stands for unspecific bands of anti-Cc antibody, as reported by the manufacturer. (C) Western blots against cytosolic (tubulin), mitochondrial (Hsp60), and nuclear (H3) markers. (D) Subcellular localization of HIGD1A and Cc proteins by confocal microscopy. Heltog cells were transfected to express the chimera HIGD1A-mCherry (red; first column of *Upper* and *Middle* rows). The chimera Cc-GFP is stably expressed in the Heltog cell line (green; second columns). Nuclei were stained in blue with Hoechst (third columns) and mitochondria with MitoTracker Red CMXRos (red, first panel of *Lower* row). Colocalization between green Cc-GFP and red HIGD1A-mCherry in mitochondria, along with blue nuclear staining, is shown in the merge images (last columns of *Upper* and *Middle* rows). (Scale bars: 10  $\mu$ m.)



**Fig. 2.** ITC assays of CcO and HIGD1A with WT and Y97pCMF Cc. (A and B) Thermograms (Upper) and binding isotherms (Lower) of WT Cc/CcO (A) and Y97pCMF Cc/CcO (B) complexes in their reduced states. Experimental data were fitted to a 2:1 model with the same  $K_D$  values. (C and D) Thermograms (Upper) and binding isotherms (Lower) of WT Cc/MBP-HIGD1A (C) and Y97pCMF Cc/MBP-HIGD1A (D) complexes at reduced states. Data were fitted to a model with a 1:1 stoichiometry.

phosphorylation to maintain low levels of apoptotic-inducing ROS. Furthermore, Y97pCMF Cc was an inefficient caspase activator. Altogether, these findings can shed light on the function of phosphorylated Cc in insulin-stimulated postischemic neuroprotection.

## Results and Discussion

**Substitution of Tyr97 with pCMF Allows for Study of Cc Phosphorylation, Preserving Its Physicochemical Properties.** To accurately study the phosphorylation of Cc at Tyr97, this residue was substituted with the noncanonical amino acid pCMF, which is an analog of phosphotyrosine residue. To do that, we used the evolved tRNA synthetase method as previously reported (SI Appendix, Fig. S1). As described above, this substitution simulates the charge and the volume of a phosphotyrosine residue better than a glutamate substitution (33).

To confirm that the noncanonical amino acid pCMF was successfully introduced at position 97 of the heme protein, the Y97pCMF Cc mutant was purified to homogeneity, and its molecular mass was compared with that of WT Cc by MALDI-TOF (SI Appendix, Fig. S2 A and B). The phosphomimetic mutant presented an increase of 41 Da in its molecular mass in

comparison with that of WT Cc, which is consistent with the expected substitution (SI Appendix, Fig. S2A). To check whether the amino acid replacement affected the secondary structure and heme environment of Cc, circular dichroism (CD) spectroscopy measurements in the far-UV and visible region were carried out, respectively. Those data demonstrated that Y97pCMF Cc exhibited an overall secondary structure and heme properties similar to those observed for WT Cc (SI Appendix, Fig. S2 C and D).

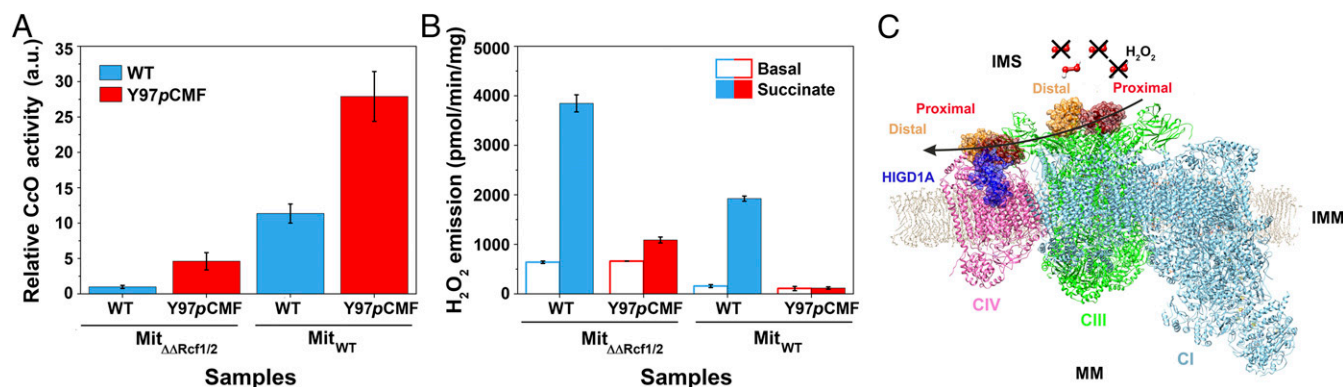
Following with the biophysical analysis of the Y97pCMF mutant, the alkaline transition phenomenon was also analyzed. This phenomenon has been related to a change in the Cc cell location, which is essential for its role in PCD (34). Such transition affects the oxidized form of Cc and involves the replacement of Met80—the sixth ligand of the heme group—by Lys72, Lys73, or Lys79 as an axial ligand at high pH values (35). To analyze the Fe-Met80( $S_6$ ) bond changes induced by pH, we therefore monitored the charge-transfer band at 699 nm, which disappears concomitantly with the alkaline transition (SI Appendix, Fig. S3A) (36). The quantitative analysis yields a difference of  $\sim 0.8$  in the  $pK_a$  values for this alkaline transition in the WT and Y97pCMF Cc species ( $pK_{a\text{-WT}} = 8.9 \pm 0.4$  and  $pK_{a\text{-Y97pCMF}} = 8.1 \pm 0.2$ ; SI Appendix, Fig. S3 B–D).

On the other hand, given that most mutations in Tyr97 performed to date alter the thermal stability of Cc, we explored that possibility in Y97pCMF Cc (31, 37). In terms of secondary and overall structure, data from far-UV CD and fluorescence, respectively, showed that the midpoint melting temperature ( $T_m$ ) of Y97pCMF was slightly lower than that calculated for WT Cc (Table 1). Notably, in contrast to the data recorded for the heme group environment of WT species, the visible CD data of Y97pCMF Cc fit well into a two-state model (Table 1). Such findings could indicate a slight destabilization in the N-terminal  $\alpha$ -helix pack, which constitutes an early event in Cc folding (38–41).

Altogether, the biophysical comparison of WT and Y97pCMF Cc species indicates that the substitution of Tyr97 with non-canonical pCMF is a good approach to studying the consequences of Cc phosphorylation without significantly altering the physicochemical and structural properties.

**CcO Activity Is Modulated by Y97pCMF Cc and Respiratory Supercomplex Factors.** Given that phosphorylation of Cc has been described as a phenomenon that modulates the functions of this metalloprotein, we explored the behavior of Y97pCMF Cc as an electron donor. The in vitro ability of Y97pCMF Cc to reduce CcO was measured in the absence or presence of HIGD1A, which acts as a positive modulator of CcO (29). In the absence of a modulator, the CcO activities were slightly higher with the Y97pCMF mutant compared with the WT Cc (Fig. 1A). Then we wondered whether those slight changes were due to a change in the molecular recognition mode between both Cc species and CcO. Thus, we analyzed the interaction between CcO and WT or phosphomimetic Y97pCMF Cc by isothermal titration calorimetry (ITC) (Fig. 2A and B). Both isotherms were fitted to a model with two independent binding sites, as previously described for the cross-complex between human WT Cc and bovine CcO (42). Notably, the  $K_D$  values showed that Y97pCMF Cc had a higher affinity for CcO than did the WT species. These results would explain the higher CcO activity shown by the phosphomimetic mutant.

Recently, it has been described that the HIGD1A-dependent increase in CcO activity was higher with a Cc phosphomimetic mutant at position 48 (9). For this reason, we tested the effect of the HIGD1A in CcO activity through WT and Y97pCMF Cc. To avoid the solubility problem of the membrane protein HIGD1A, we used a chimera with a maltose binding protein (MBP) tag in its N terminus (MBP-HIGD1A) (SI Appendix, Fig. S4). Notably, CcO activity significantly increased upon addition of MBP-HIGD1A through Y97pCMF Cc (Fig. 1A). To discard a possible contribution from



**Fig. 3.** Effect of the respiratory factors on CcO activity and ROS production in isolated mitochondria. (A) CcO activity was measured in isolated mitochondria with added WT Cc (blue bars) or Y97pCMF Cc (red bars). Data were normalized with respect to the activity with WT Cc in mitochondria isolated from  $\Delta\Delta Rcf1/2$  yeast strains. Data represent the mean  $\pm$  SD of three independent experiments. a.u.: arbitrary units. (B) H<sub>2</sub>O<sub>2</sub> production in isolated Mit<sub>ΔΔRcf1/2</sub> and Mit<sub>WT</sub>. ROS generation was triggered by addition of succinate. Bar color code is the same as in A. (C) Molecular model of OxPhos and ROS production by respirasome when Cc is phosphorylated. The CcO-driven oxidation rate of Cc is facilitated by Tyr-97 phosphorylation and supercomplex formation to maintain low levels of apoptotic-inducing ROS. Blue, CI; green, dimeric CIII; and magenta, CIV. Cc molecules at the proximal sites of one monomer of CIII or CIV are in red, whereas those at the distal sites are in yellow. HIGD1A, colored in dark blue, makes contacts with CIV and the molecule of Cc located at its proximal site. The black arrow indicates the channeling of Cc molecules between CIII and CIV. IMM, inner mitochondrial membrane; IMS, intermembrane mitochondrial space; MM, mitochondrial matrix.

the MBP tag in CcO activity, assays were also performed with MBP protein in the presence of both Cc species. As shown in *SI Appendix, Fig. S5A*, MBP did not alter CcO activity.

Taking into account the differences observed in Fig. 1A, we would expect an eventual interaction between Cc and HIGD1A. To explore this further, we examined the interaction between MBP-HIGD1A and WT or Y97pCMF Cc by ITC (Fig. 2C and D). These measurements revealed that both Cc species interacted with MBP-HIGD1A with a stoichiometry of 1:1, yielding dissociation constant ( $K_D$ ) values within the micromolar range. To demonstrate that the interaction with the chimera was uniquely due to the HIGD1A module, we performed an ITC assay between WT Cc and the MBP protein (*SI Appendix, Fig. S5B*). These data revealed the absence of any interaction between both proteins. In addition, dilution ITC experiments showed that the observed changes in heat rate are rather due to the interaction between the Cc species and MBP-HIGD1A (*SI Appendix, Fig. S6A and B*). The redox state of Cc did not affect its interaction with HIGD1A (*SI Appendix, Fig. S6C*). Since the N-terminal domain of HIGD1A—hereafter, HIGD1A<sub>1-26</sub>—has been reported to retain Cc in the intermembrane mitochondrial space (IMS) and to decrease caspase activation (43), a set of ITC titrations were run. Data resulting from such titrations evinced that both Cc species interacted with HIGD1A<sub>1-26</sub> with similar stoichiometry values, although Y97pCMF Cc showed less affinity than WT Cc (*SI Appendix, Fig. S7*). Thus, the lesser affinity of Y97pCMF Cc toward HIGD1A<sub>1-26</sub> or MBP-HIGD1A may point out a higher interaction turnover of the phosphomimetic mutant in comparison with the WT species, thus facilitating CcO reduction (Fig. 1A).

To test the interaction between HIGD1A and Cc in cells, coimmunoprecipitation (Co-IP) assays were performed in Heltog cells—HeLa cells constitutively expressing green fluorescent protein (GFP)-tagged Cc—transfected with HIGD1A-mCherry vector. Immunoblotting data against HIGD1A corroborated the interaction between Cc and HIGD1A (Fig. 1B). The total lysate used in the Co-IP assays contained cytosolic and mitochondrial fractions, as shown by the detection of tubulin and Hsp60, respectively (Fig. 1C). Furthermore, we checked that both Cc and HIGD1A were in the same cellular compartment by confocal analysis of Heltog cells upon transfection with HIGD1A-mCherry vector, which codes for red fluorescence-tagged proteins. The pictures showed that Cc-GFP (green fluorescence) colocalized with HIGD1A-mCherry (red fluorescence)

under homeostatic conditions (Fig. 1D, *Upper and Middle*). The mitochondrial localization of Cc-GFP was confirmed by codection with the MitoTracker (Fig. 1D, *Lower*). Altogether, these findings revealed contacts between HIGD1A and Cc species.

Based on these results, the HIGD1A- and Cc phosphorylation-mediated regulations of CcO activity were tested in a cellular context using isolated mitochondria from yeast cells grown on a respiratory media. Under such growth conditions, Rcf1—a yeast ortholog of human HIGD1A—and Rcf2 stabilized the CIII–CIV interaction, favoring the formation of the supercomplexes (13, 14, 44). To analyze the effect of supercomplex formation, we used mitochondria obtained from WT and Rcf1/Rcf2-deficient yeast strains (Mit<sub>WT</sub> and Mit<sub>ΔΔRcf1/2</sub>, respectively). The supercomplex formation was impaired in Mit<sub>ΔΔRcf1/2</sub> due to the absence of Rcf1 and Rcf2 proteins (*SI Appendix, Figs. S5C and S8*). In fact, CcO activity assays using Mit<sub>ΔΔRcf1/2</sub> revealed an electron flow significantly lower in comparison with those observed using Mit<sub>WT</sub>, regardless whether WT or Y97pCMF Cc was added (Fig. 3A). In addition, a single deletion of either Rcf1 or Rcf2 partially affected the CcO activity levels, indicating a cooperative effect when the two membrane proteins Rcf1 and Rcf2 were present (*SI Appendix, Fig. S5C*). Notably, the Y97pCMF Cc mutant exhibited higher CcO activity than WT Cc, both in WT (Mit<sub>WT</sub>) and in mutant mitochondria (Mit<sub>ΔRcf1</sub>, Mit<sub>ΔRcf2</sub>, and Mit<sub>ΔΔRcf1/2</sub>) (Fig. 3A and *SI Appendix, Fig. S5C*). This finding indicates that the Rcf-mediated ETC flow is more efficient when Y97pCMF Cc participates (Fig. 3A). This suggests a double regulation mechanism through Cc phosphorylation and supercomplex formation to make an accurate cellular response against hypoxia or other respiratory stresses and needs.

**Y97pCMF Cc Impairs Mitochondrial Supercomplex-Mediated ROS Production.** Changes in the ETC flow can affect the hyperpolarization of mitochondrial inner membrane and ROS production (45). For this reason, we investigated if the accelerated CcO-driven oxidation rate of phosphorylated Cc could alter mitochondrial ROS production both in the absence and in the presence of respiratory supercomplexes. ROS production in mitochondria from *Saccharomyces cerevisiae* occurs mainly in CIII as its mitochondria are deficient in CI (46, 47). Significant contribution of complex II (CII) to ROS generation has recently been described in mammalian mitochondria (48–50). In *S. cerevisiae*, however, contribution of CII to ROS generation occurs only when specific mutations are present near the proximal quinone-binding

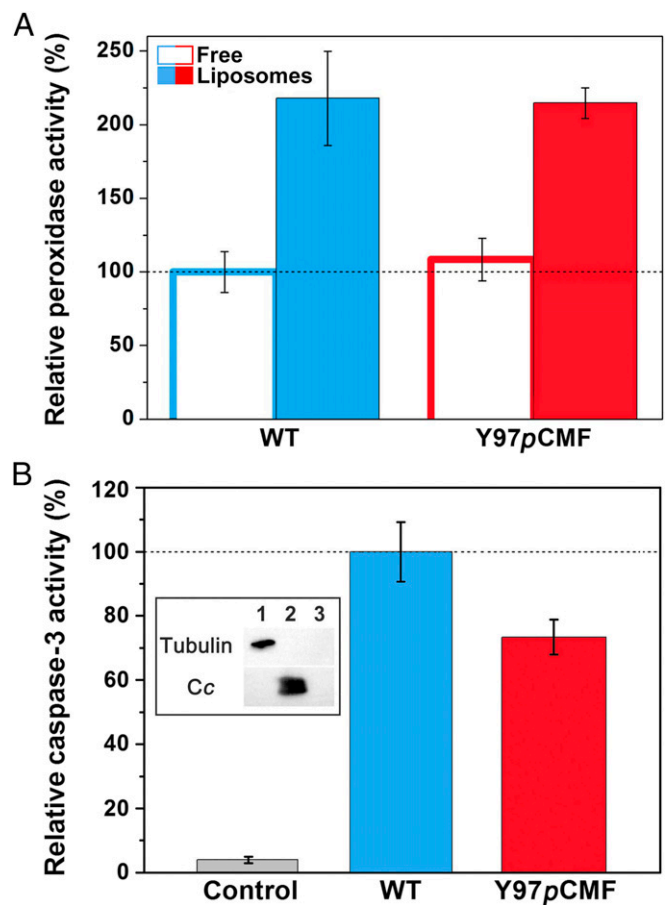
site ( $Q_p$ ) of CII (51). Since our study was focused on Cc-mediated ROS production, with Cc acting as an electron donor between CIII and CIV, no ETC inhibitors were used. Therefore, succinate was added as electron donor to both types of mitochondria, in the presence of either WT or Y97pCMF Cc, to trigger the ETC through CII. Upon addition of succinate, ETC-dependent  $H_2O_2$  production significantly increased, especially in Mit $_{\Delta Rcf1/2}$  where the formation of supercomplexes is impaired (Fig. 3B and *SI Appendix*, Fig. S9). Remarkably, Y97pCMF Cc made ROS production decrease in Mit $_{\Delta Rcf1/2}$  and, more pronouncedly, in Mit $_{WT}$  where supercomplexes are formed (Fig. 3B). These findings suggest that the CcO-driven oxidation rate of Cc is tightly regulated by Cc phosphorylation and supercomplex formation to keep apoptotic-inducing mitochondrial ROS at a low level.

Thus, we propose a respirasome OxPhos model based on the recently described extra distal Cc-binding sites in CIII and CIV, thus providing a path for diffusion of Cc molecules (Fig. 3C) (42, 52). This way of communicating between complexes through Tyr97-phosphorylated Cc could accelerate the electron transfer flow, which, in turn, would avoid ROS-mediated cellular damage to guarantee neuronal survival during ischemia reperfusion in the brain (28, 45).

**ROS Scavenger Activity and Cardiolipin Binding of Cc Remains Unaltered upon Phosphorylation.** We wondered whether the low levels of ROS displayed by the presence of Y97pCMF Cc might be caused by an exacerbated peroxidase activity of the heme protein. We thus measured such enzymatic activity in WT and mutant Cc, either free or bound to lipid vesicles containing CL at a 4:1 ratio [1,2-dioleoyl-*sn*-glycero-3-phosphocholine:1,1',2,2'-tetraoleoylcardiolipin (DOPC:TOCL)]. No differences in peroxidase activity were observed between Y97pCMF and WT Cc species, both when free or bound to liposomes. It should be noted that the use of liposomes rose the peroxidase activity considerably, although in the same way for WT and phosphomimetic Cc (Fig. 4A). This finding corroborates that the drop in ROS production upon mitochondrial supercomplex assembly is due to a more efficient CcO-driven oxidation rate by Y97pCMF than by WT Cc.

In addition, the onset of PCD is triggered by the ability of the heme protein to leave the mitochondria after binding to CL (53). Therefore, a plausible hypothesis is that the decrease in neuronal ischemic death may be due to a less efficient release of phosphorylated Cc to the cytosol, concomitantly with its role as a PCD inducer. Hence, we examined the binding of Cc species to liposomes containing CL (DOPC:TOCL) (4:1) or not (DOPC) by electrophoretic mobility shift assays (EMSA) in native agarose gels (*SI Appendix*, Fig. S10). The electrophoretic mobility of Y97pCMF and WT Cc species shifted in the same manner upon DOPC:TOCL liposome additions, unveiling that both Cc species were able to bind to CL (*SI Appendix*, Fig. S10). Likewise, EMSA data showed that none of the Cc species had altered the electrophoretic mobility with DOPC liposomes, corroborating that the two Cc species specifically interact with CL and not with other lipids of the inner mitochondrial membrane. To sum up, these data showed that there is no evidence that phosphorylated Cc at Tyr97 is preferably retained in the mitochondria.

**Y97pCMF Cc Is an Inefficient Caspase Activator.** In the context of ischemic brain damage, inhibition of both Cc–Apaf1 interaction and caspase-3 cleavage constitutes a potent neuroprotective strategy (54, 55). The binding assays involving Cc, HIGD1A, and CL suggested that both WT and Y97pCMF Cc species did seem to be equally retained in mitochondria. Therefore, the neuroprotective role of phosphorylated Cc cannot be fully explained by a specific sequestration of Y97pCMF Cc in the IMS. Thus, we explored whether phosphorylation at Tyr97 modulated Cc ability to trigger the caspase cascade in the cytosol, where the heme protein interacts with Apaf1 to assemble the so-called apoptosome.



**Fig. 4.** Peroxidase and caspase activities of Y97pCMF Cc. (A) Peroxidase activity of WT Cc (blue) and Y97pCMF Cc (red) either free (empty bars) or in the presence of liposomes containing DOPC:TOCL (4:1) (filled bars). Data represent the mean  $\pm$  SD of three independent experiments and are normalized to the peroxidase activity of free WT Cc. (B) Caspase-3 activity upon addition of exogenous WT (blue) or Y97pCMF (red) Cc. Caspase auto-activation, in the absence of added Cc, was also tested (control bar). Data represent the mean  $\pm$  SD of three independent experiments and are normalized with respect to the activity determined with WT Cc. (Inset) Western blots confirmed the lack of endogenous Cc in the cytoplasmic cell extracts by immunoblotting with anti-tubulin (cytosolic marker) and anti-Cc antibodies. Lane 1, cytoplasmic cell extracts; lane 2, purified Cc; and lane 3, BSA as a negative control.

We analyzed the caspase-3 activation driven by WT and Y97pCMF Cc, using cytoplasmic extracts of human embryonic kidney 293 cells that lack Cc (Fig. 4B). The Y97pCMF Cc-mediated caspase-3 activity decreased by about 26% with respect to that mediated by WT Cc (Fig. 4B). These findings agree with our previous results on Y97E and Y97F Cc mutants, as well as with the nitrated mono-Tyr97 Cc, which have a caspase activation slightly lower than that of the WT species (31, 56). Interestingly, none of these Cc species disrupted the Cc/Apaf1 complex assembly, and this is also expected for the Y97pCMF form. However, the formation of a nonfunctional apoptosome upon posttranslational modifications of Cc cannot be excluded (31, 56, 57).

Altogether, our data suggest that both low oxidative stress in mitochondria and less activation of the caspase cascade in cytoplasm explain neuronal survival under ischemia damage in cells containing Tyr97-phosphorylated Cc.

## Materials and Methods

Expression of genes in *Escherichia coli* and purification of Cc and HIGD1A protein constructs followed standard procedures. All experimental conditions are further available in *SI Appendix*, including strains, culture

conditions, cell transfection experiments, cell-extract fraction analyses, thermal analyses, and enzymatic activity assays. All biological samples were obtained from bacteria, yeast, or human cell extracts in full compliance with University of Seville Ethical Committee bylaws.

**ACKNOWLEDGMENTS.** We thank Prof. P. G. Schultz (Scripps Research Institute) for providing the plasmid pEVOL/pCMF/tRNA; Prof. Peter Rehling (University Medical Center Göttingen) for the antibodies  $\alpha$ Rcf1 and  $\alpha$ Rcf2; and Dr. Rosemary A. Stuart (Klingler College of Arts and Sciences, Marquette University) for the *S. cerevisiae* strains WT (*W303-1A mata leu2 trp1 ura3 his3 ade2*), RCF1::HIS3 ( $\Delta$ rcf1) (*W303-1A mata leu2 trp1 ura3 ade2*), RCF2::KAN

( $\Delta$ rcf2) (*W303-1B Mata leu2 trp1 ura3 his3 ade2*), and RCF1::HIS3 RCF2::KAN ( $\Delta$ rcf1  $\Delta$ rcf2) (*W303-1A mata leu2 trp1 ura3 ade2*). This work was supported by Ministry of Economy and Competitiveness and European Regional Development Fund (MINECO/FEDER) Grant BFU2015-71017/BMC, European Union; the Ramón Areces Foundation, European Social Fund, Andalusian Government (BIO-198); Consejo Superior de Investigaciones Científicas Fellowship JaePre\_2011\_01248 (to A.G.-C.); a Fundación Cámara PhD Fellowship (to C.A.E.-R.); Biointeractomics Platform (cicCartuja, Seville); and TA Instruments. The INSTRUCT platform of Baculovirus expression (expression screening@OPPF) was used for cloning the HIGD1A into the pOPINM vector.

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