**Isoform-specific Interaction of C-RAF with Mitochondria**

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The proteins of the RAF family (A-RAF, B-RAF, and C-RAF) are serine/threonine kinases that play important roles in development, mature cell regulation, and cancer. Although it is widely held that their localization on membranes is an important aspect of their function, there are few data that address this aspect of their mode of action. Here, we report that each member of the RAF family exhibits a specific distribution at the level of cellular membranes and that C-RAF is the only isoform that directly targets mitochondria. We found that the RAF kinases exhibit intrinsic differences in terms of mitochondrial affinity and that C-RAF is the only isoform that binds this organelle efficiently. This affinity is conferred by the C-RAF amino-terminal domain and does not depend on the presence of RAS GTPases on the surface of mitochondria. Finally, we analyzed the consequences of C-RAF activation on mitochondria and observed that this event dramatically changes their morphology and their subcellular distribution. Our observations indicate that: (i) RAF kinases exhibit different localizations at the level of cellular membranes; (ii) C-RAF is the only isoform that directly binds mitochondria; and (iii) through its functional coupling with MEK, C-RAF regulates the shape and the cellular distribution of mitochondria.

RAF kinases are a family of Ser/Thr kinases (A-RAF, B-RAF, and C-RAF) that are found upstream of the highly conserved mitogen-activated protein kinase (MAPK) signaling module, MEK-ERK. As such, they link ERK activation to different growth signals (1). Mutations that confer a constitutively active status to the protein B-RAF are among the most commonly detected genetic abnormalities in human carcinogenesis (1). The variety of defects observed upon the knock-out of each of the RAF genes indicate that they play both overlapping and distinct roles in mouse development. However, it is unclear to which extent this can be attributed to their tissue-specific expression pattern and/or to specific roles that these kinases might exert at the cellular level (2–4).

A distinctive property of the RAF kinases is their ability to interact with cellular membranes. This localization imparts distinct properties to RAF kinases in terms of signaling; the small GTPases of the RAS family, which are membrane-anchored proteins, link RAF kinases to activated membrane receptors (5). Compared with the cytosol, cellular membranes also contain a reduced content in phosphatases, thereby increasing the functional output of C-RAF activation when this kinase is located on membranes (6). RAF kinases interact not only with the plasma membrane; C-RAF is also present on the surface of mitochondria (7–12). Although the molecular mechanisms and the consequences of this localization are far from being uncovered, all reports to date mention that C-RAF exerts anti-apoptotic and pro-oncogenic effects at this level (9, 10). The existence of target proteins for RAF kinases on the surface of mitochondria, such as the protein BAD, a “BH3-only” member of the Bcl family (7, 13), suggests that these kinases could control directly some important aspects of the physiology of these membrane organelles.

Despite the potentially important consequences of RAF localization to cell membranes, many important questions remain to be addressed. First, do all RAF isoforms exhibit identical or distinct membrane tropism? Considering the interaction of RAF kinases with mitochondria, it remains unclear whether C-RAF is the only isoform that is present at this level. What are the structural determinants of RAF involved in mitochondrial interaction, and what type of molecules are recognized? How is the interaction of RAF kinases with mitochondria regulated? What are the consequences in terms of mitochondrial physiology? Here, we have used different experimental strategies to address these questions. Our findings reveal the specificity of RAF interaction with cellular membrane organelles and shed light on their mode of action at this level.
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EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The cell lines used in this study were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, 2 mM l-glutamine, and antibiotics (penicillin-streptomycin), with the exception of HUVECs (PromoCell), which were grown in an endothelial basal growth medium supplemented with 20% fetal bovine serum, 20 mg/ml basic fibroblast growth factor, 10 ng/ml epidermal growth factor (Invitrogen), and 1 μg/ml heparin (Sigma). Mouse embryonic fibroblasts (MEF) immortalized with the SV40 large T antigen (clones K2 (C-RAF−/−) and K9 (C-RAF+/+)) were obtained from C. Pritchard (University of Leicester, United Kingdom) (14). Mice of the FVB strain used in these studies were kept and anaesthetized according to protocols approved by the animal care and use committee at the University of Würzburg.

The primers and strategy used for the construction of all RAF expression vectors, as well as the antibodies used in this study, are described in the online supplemental material. Rabbit reticulocyte lysates were synthesized and radiolabeled with [35S]Cys/Met (Redivue-Cys-Met Mix, GE Healthcare) using the reticulocyte lysates were synthesized and radiolabeled with [35S]Cys/Met (Redivue-Cys-Met Mix, GE Healthcare) using the TNT T7 quick-coupled transcription-translation system (Promega) according to the manufacturer’s instructions. Standard protocols were used for immunoblotting and enhanced chemiluminescence detection of the proteins (ECL kit from Pierce).

Immunofluorescence—Immunofluorescence labeling was performed according to conventional protocols. For experiments presented in Figs. 1 and 4, cells were transfected with vectors encoding untagged versions of each RAF isoform according to standard procedures (Lipofectamine 2000, Invitrogen). After overnight expression, we performed a prepermeabilization step; the cells were treated with digitonin (50 μg/ml) in a buffer containing 10 mM KCl, 1.5 mM MgCl2, 1 mM EGTA, 1 mM EDTA, 250 mM sucrose, 20 mM Heps-KOH, pH 7.4, with protease inhibitors for 4 min on ice. The cells were subsequently fixed and processed for immunofluorescence. Pictures were acquired on a TCS SP2 confocal microscope (Leica) equipped with a 63× HCX PL APO, NA = 1.40 objective (Leica), under oil immersion. Following acquisition, the images were combined using Photoshop software (Adobe).

Video microscopy—HUVECs were electroporated with the mitochondrial fluorescent marker DsRed-mito (Clontech). Cells were filmed for 2 h under constant conditions of 5% CO2 and 37 °C and observed by UV-lamp using an Axiovert 200 microscope equipped with shutter-controlled illumination (Carl Zeiss) and a cooled digital charge-coupled device camera (Roper Scientific) using a ×40 lens. Images were processed using MetaMorph 2.0 image analysis software (Universal Imaging) and QuickTime pro 7 software (Apple Computers).

Mitochondrial Purification—Mitochondria were purified from different sources according to a protocol detailed in the supplemental “Materials and Methods.” The analysis of different markers revealed that this preparation was devoid of plasma membrane (E-cadherin), nuclear (histone H3), and cytoskeletal markers (tubulin) but contained membranes from the endoplasmic reticulum (Gpr98) and endosomes (Rab7) (supplemental Fig. 1A). The mitochondria retained their functionality (polarity and ability to import intrinsic preproteins) after isolation (data not shown).

In Vitro Assay for Mitochondrial Binding and Import—For each mitochondrial binding reaction, we mixed 50 μg of mitochondrial proteins with 10 μl of reticulocyte lysate in a binding buffer consisting of 55 mM manitol, 18 mM sucrose, 80 mM KOAc, 8 mM MgCl2, 1 mM dithiothreitol, 20 mM Heps-KOH, pH 7.4, and 0.5 mg/ml bovine serum albumin. Unless stated otherwise, the samples were incubated for 15 min on ice. After a brief centrifugation, the mitochondrial pellets were rinsed, resuspended, and resuspended in sample buffer. Following separation on SDS-PAGE, radioactivity was detected with the autoradiography enhancer En3hance (PerkinElmer Life Sciences) with ECL films (Amersham Biosciences). The results were quantified using NIH ImageJ software and presented as the percentage of input bound. To test their ability to import RAF kinases, mitochondria were incubated with RAF kinases in the same buffer with 5 mM succinate, 1 mM ATP, and 0.08 mM ADP. The import reactions were performed at 30 °C for 20 min. Following the reisolation of mitochondria, proteinase K was applied on ice for 10 min (10 μg/ml final concentration). Proteolysis was stopped with addition of phenylmethylsulfonyl fluoride, and the samples were analyzed by autoradiography as described previously.

Mitochondrial Treatments—For trypsinization, to remove proteins from the surface of mitochondria, preparations of this purified organelles were incubated with 20 μg/ml trypsin for 15 min on ice in storage buffer. Proteolysis was stopped with the addition of 500 μg/ml soybean trypsin inhibitor, and mitochondria were resuspended and used for binding assays. For EDTA treatment, mitochondria were pretreated for 5 min with 20 mM EDTA and later incubated with lysates, again in the presence of EDTA.

Electron Microscopy—Cells grown on coverslips were fixed for 45 min with 2.5% glutaraldehyde (50 mM cacodylate, pH 7.2, 50 mM KCl, 2.5 mM MgCl2) at room temperature, fixed for 2 h at 4 °C with 2% OsO4 buffered with 50 mM cacodylate (pH 7.2), washed with H2O2, and incubated overnight at 4 °C with 0.5% uranyl acetate. The cells were dehydrated, embedded in Epon 812, and ultrathin sectioned. The sections were analyzed with a Zeiss EM900 (Carl Zeiss).

Anthrax Lethal Toxin (LT) Purification and Use on Cells—The two components of LT, protective antigen (PA) and lethal factor (LF), were produced and purified separately as recombinant proteins in Escherichia coli. The genes encoding LF and PA were PCR-amplified from genomic DNA of Bacillus anthracis strain Sterne (a gift from Patrice Boquet, Nice, France) and cloned into pQE30 (Qiagen) and pET22b (Novagen) expression plasmids. Both proteins were obtained in a His6-tagged form and purified by immobilized metal affinity chromatography (IMAC, Chelating Fast Flow, Amersham Biosciences). PA was further purified on a mono-Q column (Amersham Biosciences). Following their purification, the proteins were concentrated and dialyzed in 25 mM Tris-HCl, pH 7.4, 250 mM NaCl. PA and LF were simultaneously applied on cells at final concentrations of 3 and 1 μg/ml respectively, in normal culture medium.
RESULTS

RAF Kinases Exhibit Distinct Localizations on Cellular Membrane Organelles—Although the literature contains multiple fragmentary reports on the ability of RAF kinases to interact with cellular membranes, no comparison has yet been performed among all isoforms. To analyze simultaneously the membrane localization of all isoforms of RAF kinases, we devised a strategy intended to overcome the following technical obstacles: (i) RAF kinases exhibit non-overlapping tissue-specific expression patterns and are generally expressed at low endogenous levels; (ii) RAF kinases tend to heterodimerize under growth conditions (15–17), rendering the analysis of individual isoform behavior difficult; (iii) each RAF kinase is present predominantly as a cytoplasmic pool. To compare directly the membrane localization of each RAF isoform, we overexpressed them and applied conditions of serum starvation. Then, immediately before the coverslips were fixed and processed, we performed a mild permeabilization in order to eliminate the cytosolic pool of the kinases. Using this strategy in HeLa cells, we noticed that each RAF kinase exhibited distinct membrane targeting properties (Fig. 1). C-RAF almost completely colocalized with mitochondria (Fig. 1, A–C). Although we also detected a strong immunoreactivity for C-RAF in the nuclei of HeLa cells, this immunoreactivity was not attributable to the presence of the full length C-RAF but rather to a short fragmentary report of the ability of RAF kinases to interact with the mitochondrial level.

C-RAF Efficiently Binds to Purified Mitochondria in Vitro—To gain more insight into the interaction of RAF kinases with mitochondria, we decided to analyze this interaction in vitro. RAF kinases were produced and radiolabeled with \(^{35}\)S/Cys/Met by in vitro transcriptions/translations in reticulocyte lysates (as initially reported by Stancato et al. (18), and mixed with purified mitochondria obtained from human embryonic kidney (HEK) 293 cells. Autoradiographic analysis revealed that the RAF isoforms exhibit different abilities to associate with mitochondria (Fig. 2B). Although C-RAF associated with mitochondria in a fast and efficient fashion, the two other isoforms exhibited a significantly reduced binding (A-RAF) or behaved like the kinase MEK, which was included as a negative control in this assay (B-RAF) (Fig. 2B). The binding efficiency of C-RAF, measured as a percentage of the input bound at 15 min, was more than 2-fold higher than A-RAF and more than 5-fold higher than B-RAF (Fig. 2C). At this stage, a contamination of our mitochondrial preparations with endosomal membranes, detected with the markers Rab7 (supplemental Fig. 1A) and EAA1 (data not shown), constituted the most likely explanation for the residual binding observed with A-RAF using mitochondria from cultured cells. We extended our analysis to mitochondria obtained from a normal tissue, in this case from

Statistics—Statistical significance was determined using a one-tailed paired Student’s \(t\) test.

The figure below shows the results of the experiments described above.
mouse liver (Fig. 2D). Again, we noticed a clear difference between C-RAF and the two other isoforms; C-RAF bound almost 5-fold more efficiently to mitochondria than B-RAF and A-RAF (Fig. 2D). We verified the specificity of our binding assay by replacing mitochondria with purified microsomal membranes (Fig. 2E) and detected no binding of C-RAF to microsomes, suggesting that the experiments presented indeed reflected C-RAF-specific interaction with mitochondria.

Because the possibility that one of the RAF kinase isoforms (A-RAF) might be partially present inside mitochondria had previously been proposed by Yuryev et al. (19), we decided to test whether RAF kinases would be imported into this organelle. We tested this possibility by performing proteolytic treatments of mitochondria that had been incubated with RAF kinases (Fig. 2F). We had observed previously that RAF kinases produced in reticulocyte lysates are sensitive to proteinase K (data not shown). Although we observed that the iron-sulfur protein Rieske was processed and imported inside mitochondria in a membrane potential-dependent fashion (abrogated by valinomycin), none of the RAF kinases demonstrated resistance to externally added protease upon incubation with mitochondria (Fig. 2F). Therefore, RAF kinases were not imported inside mitochondria in vitro. At this point of the work, we concluded that C-RAF exhibits a specific capacity to bind to the surface of mitochondria. These results strongly confirmed our initial observation that RAF kinases have different tropisms for membrane organelles and encouraged us to use our acellular system further to learn more about C-RAF interaction with mitochondria.

Mitochondrial Affinity Is an Intrinsic Property of C-RAF and the C-RAF Mitochondrial Binding Domain Localizes to Its Amino Terminus—Our observations that RAF kinases exhibit different affinities for mitochondria in acellular conditions suggested that the differences of subcellular distribution we had initially observed were intrinsic properties of the RAF kinases rather than the product of a cellular regulation that would have applied to one of the RAF isoforms. To examine the possibility that the activation of these kinases, for example obtained through the introduction of an oncogenic mutation, might interfere with their recruitment to mitochondria, we compared C-RAF and B-RAF wild type with their oncogenic versions, C-RAF DDED (mutated on residues 338, 341, 491, and 494) (20) and B-RAF V600E. We found that these mutations did not alter the mitochondrial binding properties of either C-RAF or B-RAF (supplemental Fig. 2A). We also focused our attention on the role of Ser-338, a residue in which phosphorylation had previously been proposed to constitute a mitochondrial targeting signal (9, 10). A non-phosphorylatable mutant in which this
residue was replaced with Ala was compared with C-RAF WT; we found that C-RAF S338A exhibits an affinity for mitochondria that is comparable to C-RAF WT (supplemental Fig. 2B). Reciprocally, phosphomimetic mutants with an Asp residue introduced on Ser at positions 338 and 339 did not increase the recruitment of C-RAF to mitochondria (data not shown). These findings indicated that the phosphorylation of Ser-338 is neither necessary nor sufficient for the mitochondrial targeting of C-RAF. We concluded that the conformational changes that accompany the activation of RAF kinases do not change their affinity for mitochondria.

To address directly the possibility that C-RAF affinity for mitochondria might be an intrinsic property of this isoform, we decided to identify the sequence determinants that account for it. We constructed a set of vectors allowing the expression of different parts of C-RAF, as well as some chimeras between C-RAF and B-RAF (Fig. 3). Each protein was synthesized and radiolabeled in vitro in reticulocyte lysates and incubated with purified mitochondria as described above (Fig. 3). Interestingly, all chimeras in which the first 100 amino-terminal residues of B-RAF were substituted with corresponding residues from C-RAF showed an increased recruitment to mitochondria (Fig. 3). These results indicated that the amino-terminal regulatory region of RAF kinases determines their mitochondrial affinity, an interesting point considering that this region concentrates almost all sequence divergences between the isoforms of RAF. To determine directly which part of the C-RAF kinase binds mitochondria, two constructs covering the carboxyl terminus of C-RAF (residues 324–648) or its amino terminus (residues 1–323) were compared in this assay. This analysis revealed that the amino-terminal domain of C-RAF binds efficiently to mitochondria, in contrast to its carboxyl terminus (Fig. 3). We concluded that C-RAF amino terminus confers its mitochondrial interaction properties on the entire C-RAF kinase.

Protein Binding Partner(s) Other Than RAS GTPases Support C-RAF Recruitment on Mitochondria—RAF kinases, and in particular C-RAF, can interact in vitro both with proteins and also with lipid mixtures (21). To determine which of these molecules C-RAF would recognize, we treated purified mitochondria with trypsin (Fig. 4A). This procedure, which removed the proteins of the mitochondrial outer membrane (Bcl-XL) but left intact intrinsic mitochondrial proteins (the matrix protein Mn-SOD), almost completely abolished the recruitment of C-RAF (Fig. 4A). Therefore, we concluded that C-RAF interacts with proteins of the mitochondrial surface. Small GTPases of the RAS family constitute a prime candidate as binding partners for RAF kinases on the surface of mitochondria. To test their contribution to the recruitment of C-RAF, we used the following approaches: (i) we tested the effect of Mg$^{2+}$/H$^{+}$ chelation by EDTA, a procedure that triggers the transition of small GTPases to an inactive, nucleotide-free form (Fig. 4B); and (ii) we compared the binding of C-RAF WT with the mutant R89L, a mutation that abrogates the binding of C-RAF to activated RAS (22) (Fig. 4C). Clearly, neither of these two procedures abrogated the binding of C-RAF to mitochondria (Fig. 4, B and C). We concluded that, under the conditions of our assay, protein binding partners other than RAS GTPases account for most of the binding of C-RAF to mitochondria. To confirm this observation in intact cells, we tested the intracellular distribution of the mutant R89L of C-RAF with the protocol that we had used for the experiments presented in Fig. 1. We found that C-RAF R89L exhibited the same mitochondrial localization as C-RAF WT (supplemental Fig. 3). We concluded that the mitochondrion...
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FIGURE 4. Role of mitochondrial surface proteins and of the small GTPases of the RAS family as interaction partners for C-RAF. A, mitochondrial surface proteins are required for C-RAF binding. Purified mitochondria were treated with trypsin. To monitor the selective removal of mitochondrial surface proteins by this protocol, mitochondria were analyzed by immunoblot for their content of proteins of the mitochondrial surface (Bcl-XL) or matrix (manganese-superoxide dismutase (Mn-SOD)). Trypsinized mitochondria exhibit a drastically reduced ability to bind C-RAF (*, p < 0.01 compared with control (Ctrl)). B, EDTA treatment. Mitochondria were pretreated on ice with 20 mM EDTA and used later for a binding assay with C-RAF. ns, indicates no significant difference compared with control. C, mitochondrial interaction properties of the mutant C-RAF R89L. A mutant C-RAF R89L, which abolishes the interaction of C-RAF with RAS GTPases, was compared with C-RAF WT for its ability to bind mitochondria in vitro.

Active C-RAF Specifically Changes Mitochondrial Subcellular Distribution in a MEK-dependent Fashion—To analyze the consequences of C-RAF activation on mitochondria, we decided to use an inducible system, allowing a kinetic analysis of the consequences of C-RAF activation. We used a cell line stably expressing low levels of a fusion protein between an activated form of C-RAF (C-RAF-BXB) and the hormone-binding domain of the estrogen receptor (23). This oncogenic version of C-RAF, which consists of an internal deletion of its Ras-binding domain of the estrogen receptor (23), exhibits no significant difference compared with control. C-RAF activation causes an intense remodeling of this organelle, characterized by a change from long, filamentous to short, spherical mitochondria clustered around the nucleus. The activation of C-RAF was sufficient to produce this mitochondrial remodeling, because it could be observed in serum-free conditions (Fig. 5, A–D). Transmission electron microscopy confirmed these observations (Fig. 5, E and F); although mitochondria from control cells were visible as longitudinal as well as cross-sections, we observed spherical elements only in cells in which C-RAF had been activated for 12 h. Despite this intense change in shape and distribution, the inner architecture of mitochondria remained normal (Fig. 5, E and F). Mitochondrial remodeling occurred progressively upon 4-OHT addition and was systematically noticed after 12 h of treatment (Fig. 5G). At the same time, all mitochondrial markers that we tested remained at a stable level (Fig. 5H). The distribution of other membrane organelles was insensitive to C-RAF activation; early endosomes, labeled with the fluorescent marker GFP-membrane organelles was insensitive to C-RAF activation; remained at a stable level (Fig. 5).

At the same time, all mitochondrial markers that we tested remained at a stable level (Fig. 5).

To relate directly the ability of C-RAF to remodel and relocate mitochondria with the property of this kinase to interact with these organelles, we compared the distribution of mitochondria in NIH3T3 cells expressing an active version of C-RAF (C-RAF DDED) with different mutants that either abolished mitochondrial binding (C-RAF DDED ΔN), a deletion of C-RAF amino terminus) or strictly confined its localization to mitochondria (the mutant M-RAF, a fusion of C-RAF BXB with the mitochondrial localization determinant of the protein Tom70, reported previously by Wang et al. (7). All constructs were expressed at a high level and were functional kinases based on the increased levels of phospho-ERK that we measured upon

Changes in mitochondrial morphology, such as fragmentation and perinuclear clustering, are common findings in cells that undergo apoptosis or the loss of mitochondrial membrane potential. To rule out the contribution of a form of toxicity, we checked that mitochondria from these cells retained cytochrome c and a normal uptake of MitoTracker, a dye in which uptake is sensitive to ΔΨm (supplemental Fig. 5), suggesting that these organelles had kept their functionality. In addition, we verified that the phenotype induced by C-RAF activation was not abolished in the presence of benzyloxy-carbonyl-VAD-fluoromethyl ketone (Z-VAD-fmk), a broad spectrum inhibitor of caspases (data not shown). To analyze the contribution of the kinase MEK, the classical effector for the kinases of the RAF family, we used two different inhibitory approaches: the chemical inhibitor U0126 and the bacterial toxin LT from B. anthracis. LT is a protease that cleaves MAPKK at their amino terminus in a potent and specific fashion (24, 25). This cleavage reduces the affinities of MEKs for their substrate ERKs as well as their intrinsic kinase activity (25). Compared with the other approach that can be used to inhibit the kinase MEK, LT exhibits two important advantages: (i) its specificity is conferred by the enzymatic nature of its activity, and (ii) LT affords a direct monitoring of the inhibition of MEK through the visualization of an electrophoretic mobility shift consequent to its cleavage (supplemental Fig. 6). In our hands, both U0126 and LT resulted in a complete inhibition of the mitochondrial remodeling induced by activated C-RAF (Fig. 5I). The transfection of a dominant-negative form of the kinase MEK (MEK LIDA) also prevented this remodeling (supplemental Fig. 7). We concluded that the kinase MEK is an important effector of C-RAF with respect to the regulation of mitochondrial shape and distribution.

C-RAF Regulation of Mitochondrial Subcellular Distribution Correlates with the Ability of C-RAF to Interact with This Organelle—To relate directly the ability of C-RAF to remodel and relocate mitochondria with the property of this kinase to interact with these organelles, we compared the distribution of mitochondria in NIH3T3 cells expressing an active version of C-RAF (C-RAF DDED) with different mutants that either abolished mitochondrial binding (C-RAF DDED ΔN), a deletion of C-RAF amino terminus) or strictly confined its localization to mitochondria (the mutant M-RAF, a fusion of C-RAF BXB with the mitochondrial localization determinant of the protein Tom70, reported previously by Wang et al. (7). All constructs were expressed at a high level and were functional kinases based on the increased levels of phospho-ERK that we measured upon...
their intracellular expression (supplemental Fig. 8). Although the expression of C-RAF DDED led to a clear mitochondrial remodeling consisting in the disappearance of all filamentous aspects (Fig. 6, A–C), neither an activated A-RAF (A-RAF Y299D/Y300D) (data not shown) nor C-RAF DDED/H9004N exerted any such effect (Fig. 6, D–F), suggesting that the mitochondrial binding ability of C-RAF was required for the remodeling. This point was further confirmed by observing cells that

FIGURE 5. C-RAF activation changes mitochondrial subcellular distribution. A–D, NIH3T3 cells stably expressing low levels of an activable version of C-RAF (C-RAF BXB-ER fused with the hormone-binding domain of the estrogen receptor) were used to analyze kinetically the effects of C-RAF activation on mitochondria. Cells placed in conditions of serum starvation were incubated either with 1 μM 4-OHT for 12 h or a vehicle. The pictures are representative of findings obtained upon confocal examination after staining for the mitochondrial marker Tom20 (green) and nuclei (4,6-diamidino-2-phenylindole, blue) without (A and C) or following C-RAF activation (B and D). In C and D, we present close-ups corresponding to the area enclosed by dotted line boxes in A and B. E and F, mitochondrial ultrastructure in control cells and after C-RAF activation. In control cells (E) the majority of mitochondria have an elongated shape, and only few mitochondria with spherical profiles are visible (indicated here with an asterisk). After 12 h of activation of C-RAF by the addition of 4-OHT (F), we noticed predominantly spherical mitochondria (bar: 0.5 μm). G, quantification of the percentage of cells with remodeled mitochondria after 0, 6, or 12 h of exposure to 4-OHT in cells expressing C-RAF BXB-ER compared with wild-type NIH3T3 cells. Results were obtained by blind counting of random microscopic fields. H, immunoblot analysis of different mitochondrial markers. Cellular extracts prepared from NIH3T3 C-RAF BXB-ER cells cultured under control conditions/exposed to 4-OHT for 12 h were compared for their content in the indicated markers. Ph-ERK, phospho-ERK; Cyt c, cytochrome c; VDAC, voltage-dependent anion channel; Mn-SOD, manganese-superoxide dismutase. I, U0126 and anthrax LT block mitochondrial remodeling induced by C-RAF activation. NIH3T3 expressing the C-RAF BXB-ER construct were treated with U0126 (10 μM) or B. anthracis LT, as described under “Experimental Procedures,” immediately before the application of 4-OHT. Both inhibitors abolished the mitochondrial redistribution induced by a 12-h treatment with 4-OHT.

FIGURE 6. Mitochondrial remodeling depends on mitochondrial binding properties of C-RAF. NIH3T3 cells were transfected with an active version of C-RAF (C-RAF DDED (A–C)), the same mutant in which the mitochondrial binding region was deleted (C-RAF DDED ΔN (D–F)), or a mutant in which an active C-RAF is fused to the constitutive mitochondrial targeting determinant of Tom70 (M-RAF (G–L)). In the experiments presented in the J–L, anthrax LT was applied immediately after transfection. After overnight expression, cells were processed for simultaneous immunolocalization of C-RAF (green) and mitochondria (cytochrome c, red) (bar: 20 μm).
expressed M-RAF; although we noticed that M-RAF was strictly localized to mitochondria (Fig. 6, G–L), the intracellular expression of this mutant of C-RAF again produced the phenotype we had noticed earlier, consisting of multiple short mitochondria clustered around the nucleus, with a remarkable potency (Fig. 6, G–I). The mitochondrial remodeling produced by M-RAF was prevented when LT was applied at the time of the transfection (Fig. 6, J–L). These findings collectively indicated that the ability of C-RAF to remodel mitochondria depends on the mitochondrial binding of C-RAF, and can be produced through a locally restricted activation of this kinase.

Relevance to the Physiological Context and Mechanism of Remodeling—To establish the relevance of our findings in a context that would not rely on the overexpression of C-RAF, we compared the distribution of mitochondria in MEF obtained from WT and C-RAF knock-out animals (14) (Fig. 7). Under standard culture conditions, MEF derived from C-RAF WT and knock-out animals exhibited subtle differences in mitochondrial shape and distribution. However, in the presence of phorbol 12-myristate 13-acetate (PMA, 100 nM), a strong protein kinase C agonist that can be used to activate C-RAF (27) (Fig. 7A), we noticed a striking difference between these cells; mitochondria from WT C-RAF cells appeared intensely fragmented and perinuclearly clustered in contrast to mitochondria from C-RAF knock-out cells, which remained long and were almost not redistributed (Fig. 7, D and F). The PMA responsiveness of the C-RAF knock-out MEF could be restored through the transfection of C-RAF (supplementalFig. 9). We concluded that our findings regarding C-RAF regulation of mitochondrial subcellular localization were therefore relevant to the physiological situation.

To obtain information regarding the mechanisms of C-RAF-mediated mitochondrial remodeling, we followed the distribution of this organelle by live cell imaging. The mitochondria of HUVECs were labeled fluorescently through the intracellular expression of the construct encoding the DsRed-mito marker. We observed that a rapid centripetal transport of these organelles upon PMA treatment preceded the progressive accumulation of spheric mitochondria (supplemental videos (Movie 1 and Movie 2); Fig. 8). The maintenance of mitochondrial morphology and dynamics has also been shown to be dependent on mitochondrial fission and fusion processes (28). To analyze whether mitochondrial fission contributes to the modulation of mitochondrial morphology upon activation of C-RAF, we performed immunofluorescence staining of HeLa cells treated with antibodies to visualize Drp1 and the mitochondrial protein Tom20 (supplemental Fig. 10). The results
clearly indicated that fission processes occurred upon treatment with PMA, as indicated by the association of Drp1 clusters at the endings of these small mitochondria (supplemental Fig. 10). At the same time, we found that the levels of the main protein mediators of mitochondrial fusion (Mfn1 and Mfn2, OPA1) and fission (Drrp1) remained constant, both in NIH3T3 cells treated with PMA and in cells with activated C-RAF BXB (+4-OHT) (supplemental Fig. 10). These findings suggested that both a centriplete transport and an increased ratio of fission over fusion were involved in the mitochondrial remodeling produced by C-RAF activation.

**DISCUSSION**

Although the presence of RAF kinases at the level of cellular membranes, and particularly of C-RAF on mitochondria, has been reported in multiple instances (see Refs. 7, 9, and 10; reviewed in Ref. 12), little is known about the molecular interactions involved and the eventual specificity of RAF kinases for these organelles. Here, we performed what constitutes to our knowledge the first comparative study of the distribution of all isoforms of RAF. Using a combination of fractionation, immunofluorescence, and *in vitro* interaction assay, we observed that RAF kinases exhibit strikingly different subcellular distributions and a strong specificity for intracellular membranes. We found that C-RAF is the only isoform that interacts directly with mitochondria. Our study indicates that the differences between the RAF isoforms are intrinsic parameters for each RAF isoform that are, for example, not affected by their activation or the introduction of oncogenic mutations.

Our observation that C-RAF, but not A-RAF or B-RAF, efficiently interacts with mitochondria through the direct interaction of its amino-terminal domain with this organelle needs to be interpreted in the light of the similarities and divergences that exist between the sequences of the different RAF isoforms. The carboxyl terminus of the RAF kinases contains their kinase domains and is almost completely similar between the three isoforms, with the noticeable exception of a few amino acids that play an important role in the regulation of the kinase potency (29). The amino terminus of RAF kinases also contains some conserved sequences (the so-called “conserved region-1,” or CR1, including the RAS-binding domain and the cysteine-rich domain), although the overall degree of sequence homology is considerably lower at this level between the RAF isoforms. In particular, the RAF kinases possess an amino-terminal extension that is of variable length and concentrates almost all sequence divergences between the RAF isoforms (1). Therefore, it is perhaps not surprising that the distinct subcellular distributions of RAF kinases on membrane organelles, and on mitochondria in particular, can be ascribed to the amino terminus of these kinases. Our observations support the notion that the amino terminus of RAF kinases constitutes a domain that is specialized in the interaction with cellular membranes, working through a combination of both common and isoform-specific mechanisms.

In our attempt to define the molecular requirements for this interaction, we found that the mitochondrial localization of C-RAF does not depend on the small GTPases of the RAS family. Our findings do not rule out the possibility that the RAS GTPases might play a direct and important role in the recruitment of RAF kinases in specific situations (30, 31) or even that it might regulate specific aspects of the interaction of C-RAF with mitochondria, such as its inclusion in specialized membrane microdomains (5, 32). Nevertheless, we suggest that RAF kinases play an active role in the compartmentalization of the MEK-ERK signaling cascade on cellular membranes. In this respect, our findings provide new information regarding the regulation of the RAF-MEK-ERK cascade on cellular membranes. Until now, the GTPases of the RAS family were the only component of the cascade for which distinct, isoform-specific, subcellular distributions had been reported (33). Based on our observations, we propose that subcellular compartmentalization operates at multiple levels of the RAS-RAF-MEK-ERK axis.

What role does C-RAF play on mitochondria? One aspect may concern apoptosis regulation, as many authors have found that C-RAF is able to counteract mitochondria-mediated apoptosis (7, 9, 12). Our findings that C-RAF remodels mitochondria could provide some elements of explanation; perhaps the formation of small mitochondrial units might limit the involvement of mitochondria during the execution of apoptosis or when limited damages happen to this organelle. Although this model remains speculative at this stage, there are multiple indications that the regulation of mitochondrial shape and distribution is an aspect that is integral to the apoptotic program (34) (reviewed in Ref. 35).

Despite its anti-apoptotic function, nothing is known regarding the effects of C-RAF on mitochondrial physiology in live cells. Our findings suggesting that C-RAF locally regulates mitochondrial shape and subcellular distribution constitute a new element in this respect. We propose that C-RAF is active locally, based on the following observations: (i) in a couple of previous reports, MEK activation led to comparable changes (*i.e.* small elements being perinuclearly clustered) in the morphology of two other membrane organelles, the Golgi apparatus and the late endosomes (36–38); (ii) C-RAF activation specifically remodels mitochondria, as we observed no effect on the other membrane compartments that we tested; (iii) the effects of activated C-RAF on mitochondria do not correlate with the levels of cytoplasmic phosphorylated ERKs but with the presence of active C-RAF on this organelle (Fig. 6). Collectively, our observations suggest that C-RAF is active locally on the surface of mitochondria and regulates their subcellular distribution through a functional coupling with its downstream effector kinase, MEK.

The mitochondrial remodeling that accompanies the activation of C-RAF is likely to be of broad consequence to cellular physiology (reviewed in Ref. 39). Indeed, the ability to regulate mitochondrial shape and position is an important parameter in processes such as cell migration (40) or the metabolism of Ca²⁺ (41). Finally, morphological alterations of the mitochondrial network are observed commonly in cancer cells (42). The frequent deregulation of the RAS-RAF axis in human cancer cells may constitute an explanation for the presence of those alterations. Clearly, more work remains to be performed in order to understand better the role of C-RAF on mitochondria.
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