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Cyclophilin A as negative regulator of apoptosis by sequestering cytochrome c.

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ABSTRACT

The release of cytochrome c from the mitochondrial intermembrane space is a decisive event in programmed cell death. Once in the cytoplasm, cytochrome c is involved in the formation of the macromolecular complex termed apoptosome, which activates procaspase-9 which in turn activates downstream procaspase-3. There are increasing evidences indicating that cyclophilin A is highly expressed in many tumours and cell lines where it exerts an anti-apoptotic function. In brain tissue, which over-expresses constitutively cyclophilin A, we evidenced mixed dimers composed of cyclophilin A and cytochrome c. In a cell free system we observed that pure cyclophilin A inhibited cytochrome c-dependent procaspase-3 activation. Moreover, we evidenced cyclophilin A-cytochrome c complexes within the cytoplasm of HCT116 cells following staurosporine-induced apoptosis. Our results strongly support that, in tumour cells, cyclophilin A is able to inhibit procaspase-3 activation by sequestering cytochrome c.

KEYWORDS

Apoptosis, brain, tumour, cyclophilin A, cytochrome c, procaspase-3, phosphatidylethanolamine-binding protein.

ABBREVIATIONS

Cyt c: cytochrome c

Cyp A: cyclophilin A

PEBP: phosphatidylethanolamine-binding protein

CsA: cyclosporin A

AMC: aminomethyl coumarin

DEVD-AMC: Acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid α - (4-methyl-coumaryl-7-amide)

EDAC: N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride

1 INTRODUCTION

Signalling for apoptosis occurs through multiple pathways that are initiated either by triggering events within the cell (DNA damage, cytoskeleton disruption) or from outside the cell by ligation of death receptors [1]. Cells respond by an active process resulting in characteristic morphological changes (DNA fragmentation and membrane blebbing). The degradation of nuclear DNA is due to caspase-dependent as well as caspase-independent DNase activation [2]. The proteolytic cleavages occurring during apoptosis are mediated at least in part by a group of proteases termed caspases, which are synthesized as inactive proenzymes. Two principal mechanisms, the mitochondrial and the death receptor pathways, are able to trigger caspase activation. The intrinsic apoptotic pathway involves the permeabilization of the outer mitochondrial membrane and the release of cytochrome c (Cyt c). This hemoprotein participates to the formation of a multimeric complex termed apoptosome, which activates procaspase-9, which in turn activates downstream procaspase-3.

Cell death or survival is determined by a balance between pro- and anti-apoptotic molecules, which are under the control of extra- as well as intracellular signals. In addition, apoptosis is modulated by a set of proteins, which are not specific of programmed cell death. For instance, in differentiated and non-dividing cells such as neurons and muscular cells, molecular chaperones are critical for maintaining cellular homeostasis. The heat shock protein family (HSP), known to control protein folding by chaperone activity, was shown to down

regulate apoptosis [3]. Moreover, in tumour cells there are increasing evidences indicating that cyclophilin A (Cyp A), a cyclosporin A-binding protein with chaperone function, can participate to cell death regulation. It has been reported that Cyp A is overexpressed in hepatocellular carcinoma [4], in small and non-small cell lung cancer [5,6] in pancreatic adenocarcinoma [7], in endometrial carcinoma [8] as well as in esophageal cancer [9]. It was shown that stable RNA interference-mediated suppression of Cyp A diminishes non-small-cell lung tumour growth *in vivo* [10]. Moreover it was observed that over-expression of Cyp A, in human prostate cancer cell line, prevented hypoxia- and cisplatin-induced apoptosis, whereas small interfering RNA-based Cyp A knockdown reversed this effect [11]. A biological mechanism accounting for the anti-apoptotic effect of Cyp A was recently reported [12]. The authors evidenced, in interleukin-6-dependent multiple myeloma cells, that cyclophilins A and B support the anti-apoptotic action of signal transducer and activator of transcription 3 (Stat3).

In this paper we show that Cyp A could control cell survival at a different level. Brain tissue expresses constitutively Cyp A in high amount [13]. We evidenced in neuron subcellular extracts stable heterodimers composed of Cyp A and Cyt c. We measured the inhibiting effect of purified Cyp A on Cyt c-dependent procaspase-3 activation *in vitro*. We determined the K_d of the Cyt c-Cyp A complex and we evidenced the formation of Cyt c-Cyp A heterodimers within the cytoplasm of apoptotic cells. Our experimental results strongly support that the anti-apoptotic effect of Cyp A may be due to the ability of Cyp A to bind free cytoplasmic Cyt c.

2 MATERIALS AND METHODS

MATERIALS

Mouse monoclonal clone 7H8 2C12 anti-Cyt c antibodies were from Bioscience (Interchim, France). Rabbit polyclonal antibodies against Cyp A were from Chemicon-Upstate (Southampton, UK). Anti-phosphatidylethanolamine-binding protein (PEBP) IgG were kindly provided by Dr Françoise Schoentgen from CNRS (Orléans, France).

METHODS

Electrophoresis and Western blots.

Proteins were resolved alternatively in denaturing and semi-denaturing 15% polyacrylamide gels. The first method was conducted essentially as described by Laemmli [14] in a discontinuous buffer system. The second electrophoretic technique was performed in a continuous buffer system (25 mM Tris, 192 mM glycine, pH 8.6, 0.1% SDS). In this case the samples were treated for 3 min at 100°C in a non-reducing solubilization solution containing 5% SDS.

Proteins resolved in either semi- or in fully-denaturing gels were blotted on a PVDF membrane (Immobilon P from Millipore, Bedford, MA) with a Trans-Blot SD semi-dry apparatus from BioRad (Hercules, CA). Membranes were probed for 2 hours with either anti-Cyt c (1/5000), anti-Cyp A (1/1000), anti-PEBP (1/1000) antibodies. Then the blots were incubated with the appropriate secondary antibody (1/2000) conjugated with peroxidase.

Measurement of caspase-3 activity.

Caspase activity was assayed by measuring the cleavage of the fluorophore aminomethyl coumarin (AMC) from DEVD-AMC (Acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid α - (4-methyl-coumaryl-7-amide)) substrate [15]. We prepared S-100 cytosolic fractions from HeLa cells according to the protocol described by Liu *et al.* [16]. The cells were suspended in HK buffer (20 mM HEPES/KOH, pH 7.5, 10 mM MgCl₂, 1 mM EDTA, 1

mM EGTA) supplemented with protease inhibitors (0.1 mM PMSF, 5 µg/ml pepstatin A, 10 µg/ml leupeptin, 2 µg/ml aprotinin and 25 µg/ml ALLN). The suspension was introduced in a Mini-Bomb cell disruption chamber (Kontes, Vineland, NJ). A 15 bar nitrogen pressure was applied to the chamber for 30 min. Cell debris were eliminated by centrifugation at 15 000 g for 10 min. Then the solution was centrifuged at 100 000 g for 60 min. The S-100 supernatant was stored at -80°C.

The Cyt c concentration was determined with a MC2 (Safas, Monaco) spectrophotometer from the sodium dithionite reduced spectrum, using an absorbance coefficient $\Delta\epsilon^{414-600} 101 \text{ mM}^{-1} \text{ cm}^{-1}$.

Caspase-3 activity was measured according to the conditions described by Hampton *et al.* [17]. The activation of procaspase was conducted at 37°C. A sample of S-100 supernatant (50 µg of protein) was mixed with 1 mM dATP and 400 nM of pure Cyt c, in some experiments bovine albumin, Cyp A or PEBP were added in the medium. The final volume was adjusted to 70 µl with buffer HK. At the end of the incubation, DEVD-AMC (50 µM) was added to the medium. The AMC fluorescence was monitored with a luminescence spectrometer Perkin Elmer (Buckinghamshire, England) $\lambda_{\text{excitation}} 354 \text{ nm}$ and $\lambda_{\text{emission}} 460 \text{ nm}$. The measure was standardised with a solution of 1 µM AMC.

Determination of Kd values.

Surface plasmon resonance analysis: The kinetic parameters were determined with a Biacore 3000 instrument (GE Healthcare, Biacore AB Uppsala, Sweden)

Equilibrium chromatography method: We used the method of Hummel and Dreyer [18] with the technical improvements indicated by Berger and Girault [19].

Induction of apoptosis in HCT 116 cells:

HCT116 colon adenocarcinoma cells were grown in complete medium, i.e., RPMI 1640 supplemented with 10% calf fetal serum and 2 mM L-glutamine at 37 °C under a humidified atmosphere with 5% CO₂. When the cells were at 80% confluence apoptosis was induced by treatment with 0.3 μM staurosporine for 24 hours. The cells were disrupted under nitrogen pressure in a Mini-Bomb chamber as indicated above. The supernatant obtained by centrifugation for 30 min at 18 000 g was stored at -80°C.

Cross-linking Cyt c-protein complexes.

20 μl of 0.1 M N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC) were added to 100 μl of HCT116 cells supernatant (4 mg/ml). The reaction was developed for 1 to 3 hours at room temperature and stopped with 20 μl of 0.1 M ethanolamine. The covalent protein complexes were separated in 15% SDS-PAGE in denaturing conditions and blotted on a PVDF membrane.

3 RESULTS

3 1 Effect of Cyp A on Cyt c-dependent procaspase-3 activation

In previous experiments we evidenced the ability of Cyt c to link small proteins, which were not directly involved in the apoptotic program, while we studied proteins of brain cell cytosol. These results are detailed in supplementary file 1. Briefly, from 115 000 g brain-cell supernatant we isolated a protein fraction, termed fraction R, containing two heterodimers of Cyt c exhibiting molecular masses of 30 and 35 kDa (figure 1A). These molecular complexes were disrupted in the presence of a disulfide reducing agent (mercaptoethanol). We later identified the Cyt c-associated proteins as being respectively Cyp A and PEBP isoform-1 (figure 1B). We investigated the ability of fraction R to trigger caspase-3 activity *in vitro*, by

comparison to pure Cyt c (data not shown). We observed that fraction R was less active than Cyt c to promote the reaction, the activity being about 30 per cent of that obtained with Cyt c. This lack of activity could result of the fact that part of the Cyt c in fraction R is immobilized in molecular complexes and had no access to the caspase activation cascade. However, fraction R was a mixture of at least 15 proteins and we could not exclude the presence of inhibitors of the reaction. In consequence we decided to investigate caspase activation in the presence of purified proteins.

We purified Cyp A and PEBP from fraction R by liquid chromatography (see supplementary file 2). We measured procaspase-3 activation triggered by Cyt c *in vitro* in a cell free system. The influence of Cyp A on the reaction is indicated in figure 2A. Cyp A inhibits the reaction at low concentration. It is active in the range of 0.3 to 0.6 μM . The mean value is roughly stoichiometric with the amount of Cyt c present in the reaction (0.4 μM). Cyp A is the intracellular receptor of cyclosporin A (CsA) and exerts a peptidyl-prolyl isomerase function (PPIase). The catalysis of prolyl isomerization by Cyp A is strongly inhibited by low amounts of CsA [20]. We tested the effect of increasing amounts of CsA on Cyp A inhibition of Cyt c-dependent procaspase-3 activation. From figure 2B, CsA is not able to suppress the inhibiting effect of Cyp A, indicating that the ability of Cyp A to bind Cyt c is independent of its PPIase activity. Cyp A, in addition to its PPIase activity, is known to exert a chaperone function within cells [21], its ability to link Cyt c could result of this latter function.

Concerning PEBP we saw that it was able to inhibit procaspase-3 activation (data not shown). However, it was less efficient than Cyp A since it requires a tenfold excess (from 3 to 6 μM) to inhibit the effect of Cyt c.

3 2 Determination of the K_d of the molecular complexes with Cyt c.

The binding affinity of Cyt c for Cyp A was measured by surface plasmon resonance analysis (see supplementary file 3). The sensorgrams led to calculate a K_d value of 16.0 ± 1.3 μM , the association and dissociation rate constants being respectively 1.05 ± 0.08 $10^2 \text{M}^{-1} \text{s}^{-1}$ and 1.67 ± 0.02 10^{-3}s^{-1} . We cannot visualize the Cyt c binding on immobilized PEBP. This probably reflects an adverse effect of the chemical immobilization of PEBP. To circumvent this problem, we used an equilibrium chromatographic technique to determine the K_d values of the proteins in solution. The K_d values obtained are 17.3 ± 4.8 μM and 9.7 ± 3.8 μM respectively for the complexes Cyp A-Cyt c and PEBP-Cyt c. For Cyp A-Cyt c interaction the affinities obtained by the two methods are in a same range

We determined the concentrations of the two proteins in pig brain, Cos and Hela cell cytosols by densitometry of blots standardized with samples of pure Cyp A or PEBP. We found that CypA was abundant in the cell lines. Its concentration expressed in μM was 17.6 ± 2.4 and 14.5 ± 0.9 respectively in HeLa and Cos cells while it was about half in pig brain (8.8 ± 2.3). It was reported by others [22] that there is a two to three fold increase of Cyp A amount when comparing tumour versus normal tissues. If we assume that the concentration of Cyp A measured in the cytosol of the cell lines, is indicative of the concentration present within tumour tissues, we can conclude that Cyp A is able to combine about half of the cytosolic Cyt c since the K_d value of the complex of the two proteins is in the same range (15 to 20 μM).

In contrast we deduced that the ability of PEBP to associate Cyt c in the cell lines was very low since we found 3.1 ± 0.2 and 0.5 ± 0.1 μM of PEBP respectively in HeLa and Cos cells cytosols . However, PEBP is highly concentrated brain cytosol (10.8 ± 2.1 μM). Its ability to combine free Cyt c in this tissue is not negligible since the K_d of the complex of the two proteins is very similar.

3 3 Detection of Cyp A/Cyt c heterodimers in apoptotic HCT116 cells.

3 3 1 Expression of Cyp A in colorectal cancer.

The protein amount of Cyp A within various tumours, was investigated by Koletsky *et al.* [23]. These authors reported that Cyp A concentration in colon adenocarcinomas was twofold to threefold greater than that found in adjacent normal tissue. In our laboratory, we obtained data corroborating these results from a previous study on gene expressed in advanced colorectal cancer (see supplementary file 4).

3 3 2 Detection of Cyt c/Cyp A protein complexes in apoptotic cells.

Cell death was induced in HCT116 cell cultures by treatment with staurosporine. The induction of apoptosis by this drug was controlled both by flow cytometry following double labelling of the cells and by measuring Cyt c efflux from mitochondria (Figure 3A). A sample of the cytosol of apoptotic HCT116 cells was cross-linked with the carbodiimide reagent EDAC. The cross-linked polypeptides were then resolved in 15% polyacrylamide gel and blotted. As indicated in figure 3B, the blot shows that one protein band cross-reacts both with anti-Cyt c and anti-Cyp A antibodies. It is likely a dimer of Cyp A and Cyt c since it exhibits an apparent molecular mass of 32 kDa. Concerning PEBP we could not evidence mixed molecular complexes with Cyt c on the blots.

4 DISCUSSION

In this study we evidenced that both PEBP and Cyp A possess the property to link Cyt c with Kd values in the range of 10 to 20 μ M. This affinity for a key component of the apoptotic machinery could lead to the inhibition of apoptosis in cells overexpressing these proteins.

The PEBP protein family is a highly conserved group of proteins found in a great variety of organisms from plants to mammals [24]. PEBP-1, a 23 kDa basic protein, was

originally purified from bovine brains [25]. During our investigations we saw that PEBP was able to inhibit caspase-3 activation *in vitro*. However, it is efficient at high concentration. The affinity of the molecular complex with Cyt c is too low to lead to the formation of significant amounts of complex *in vivo* in tumour cell lines.

Cyp A belongs to a group of proteins known as immunophilins due to their affinity for immunosuppressant drugs, which are structurally conserved throughout evolution and all have PPIase activity [21]. The physiological function of Cyp A is not well understood. Besides its catalytic activity as PPIase and its inhibiting activity on calcineurin when combined with CsA, recent studies have implicated Cyp A in additional cellular functions. Some reports suggested a role for Cyp A as mediator of intercellular communication [26]. In addition it was shown that Cyp A was incorporated into the HIV type I virions and promoted infectivity by facilitating virus uncoating [27, 28]. Moreover, within the nucleus, Cyp A alone [29] or associated with the apoptosis inducing factor (AIF) [30] can induce chromatinolysis due to its nuclease activity. Finally, Cyp A has been reported to play a role of chaperone in protein folding [21].

We have shown that Cyp A is able to bind Cyt c *in vitro*, and to inhibit procaspase-3 activation targeted by Cyt c at equimolecular concentration. The K_d value of the molecular complex Cyp A-Cyt c in solution and the concentration of Cyp A in cell lines and tumours are both compatible with the formation of substantial amounts of complex within tumour cells. The presence of cross-linked molecular heterodimers of Cyp A and Cyt c in apoptotic cells confirms that these proteins can associate *in vivo*. As we indicated in the introduction, there are many examples in literature of tumour cells overexpressing Cyp A. It was later shown that elevated concentrations of Cyp A conferred an apoptotic resistance. From our results Cyp A, which can sequester Cyt c within the cytoplasm, can consequently inhibit the mitochondrial apoptotic pathway. A comparable effect was previously ascribed to HSP27, which negatively

regulates cell death by interacting with Cyt c [31]. The inhibiting effect of Cyp A do not lead to a total suppression of apoptosis since the association of Cyp A with Cyt c is an equilibrated reaction with relatively low affinity. It seems that Cyp A can exert a “buffering” effect [32] on Cyt c by controlling the amount of free Cyt c within the cytosol. As CsA, the main ligand of Cyp A, has no influence on the association between Cyt c and Cyp A, it remains to uncover active molecules able to interact specifically with the binding site of Cyt c on CypA.

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LEGENDS TO FIGURES

Figure 1

Characterization of Cyt c molecular complexes within brain fraction R.

(A): Western blot of fraction R resolved in semi- and fully-denaturing 15% PAGE. Samples contain 60 μg fraction R (lanes 1, 2, 3 and 4) and molecular weight markers (lane 5). Cyt c is labeled with specific antibodies (lanes 1 and 3). Proteins are stained with Amido Black (lanes 2, 4 and 5). The spots cross reacting with anti-Cyt c antibodies are annotated I, IIa, IIb and III. The latter corresponds to the monomeric form of Cyt c.

(B): Cross reaction of band II with anti-Cyp A and anti-PEBP antibodies. A sample of fraction R (60 μg) is solubilized and electrophoresed in semi-denaturing conditions. After blotting the blot is probed with anti-Cyt c (lane 1), anti-Cyp A (lane 2) and anti-PEBP antibodies (lane 3). It can be seen on the figure that anti-PEBP antibodies label a protein (annotated PEBP-X) exhibiting an apparent molecular mass of 45 kDa, which is due to the dimerization of PEBP.

Figure 2

Effect of purified Cyp A on procaspase-3 activation *in vitro*.

(A): effect of Cyp A on Cyt c-dependent activation of procaspase-3. Samples of S-100 cytosol from HeLa cells (50 μg protein) are incubated at 37°C with dATP (1mM) and Cyt c (400 nM) in buffer HK (as indicated in Materials and Methods), the final volume is 70 μl . The reaction is performed in the absence or in the presence of increasing amounts of purified Cyp A (from 2 to 12 $\mu\text{g}/\text{ml}$). In control experiments Cyp A is replaced by an equal amount of bovine albumin. Procaspase-3 activation is stopped after 1 or 2 hours of incubation. The synthetic peptide DEVD-AMC (50 μM) is added to the medium and the release of free AMC by

caspase-3 is followed for 10 min with a fluorimeter ($\lambda_{\text{excitation}}$ 354 nm and $\lambda_{\text{emission}}$ 460 nm). The DEVDase activity is expressed in pmol of AMC liberated per min per mg of S-100 protein. The results represent the mean value \pm standard deviation of 5 independent experiments.

(B): effect of cyclosporin A (CsA) on Cyp A inhibition of Cyt c-dependent procaspase-3 activation. The incubation mixtures are prepared as indicated in (A). In one set of experiments the activation is triggered by Cyt c alone (400 nM) and in a second set by Cyt c (400 nM) plus Cyp A (600 nM). Increasing concentrations of CsA (from 0 to 10 μ M) are added to the two series and caspase-3 activity is determined fluorometrically after 2 hours of incubation. The results are expressed as the mean value \pm standard deviation of 3 experiments.

Figure 3

Detection of Cyt c-Cyp A crosslinks following carbodiimide treatment of the cytosol of apoptotic HCT116 cells. Cell death is induced in HCT116 by incubating the cell cultures for 24 hours with 0.3 μ M staurosporine.

(A): the cells were disrupted under pressure and the 18000 g cell supernatant is prepared as indicated in Materials and Methods. A sample (30 μ g) of control (lane 1) and apoptotic cell supernatant (lane 2) is resolved in 15% PAGE then it is transferred on a PVDF membrane. The blot is probed with anti-Cyt c antibodies.

(B): A sample (150 μ g) of the 18000 g supernatant of apoptotic cells is treated for 1 hour with 0.02 M of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC) then it is electrophoresed in denaturing conditions. The western blot is probed with anti-Cyt c (lane 4) then with anti-Cyp A antibodies (lane 5). The band cross-reacting with both antibodies (labelled with an asterisk) presents a molecular mass close 32 kDa when compared to standard molecular markers (lane 3).

Supplementary file 1: Identification of proteins associated with Cyt c in brain cytosol.

We prepared a 115 000 g brain cell supernatant as previously described [*] in PEM buffer (100 mM Pipes/NaOH, pH 6.9, 1 mM EGTA, 1 mM MgSO₄). Fraction R was purified from this cytosolic extract by chromatography on hydroxyapatite (Bio-Gel HTP from BioRad). An aliquot (75 ml) of the crude supernatant was perfused on a 50 ml hydroxyapatite column (1.6x25 cm). The column was rinsed with the PEM buffer, then with 2 M KCl in PEM buffer. The first protein fraction eluted with this buffer contained Cyt c, it was termed fraction R.

When fraction R is resolved in gel electrophoresis in semi-native conditions (Figure 1A), Cyt c distributes in 3 domains annotated I, II and III on the figure. Band III, which has an apparent molecular mass of 14 kDa is the monomeric form of Cyt c. The intermediate zone is composed of two bands at 35 (IIa) and 30 kDa (IIb) respectively. Band I was not investigated in this study. As indicated on the figure, the Cyt c-protein complexes are disrupted in fully denaturing conditions in the presence of β -mercaptoethanol.

To identify the proteins associated with Cyt c we have resolved a sample of fraction R in a preparative gel run when released from mitochondria in semi-native conditions, then, the proteins present in band II were electroeluted and analysed. Two proteins exhibiting apparent molecular masses of 23 and 17 kDa were identified by sequencing internal peptides. They were respectively: phosphatidylethanolamine-binding protein isoform-1 (PEBP) [**] and cyclophilin A [***]. Within the PEBP protein family the isoforms 1, 2 and 3 present very similar amino acid sequences [****]. Though, we further analyzed a purified sample of PEBP by mass spectrometry with a MALDI TOF/TOF Proteomics Analyzer mass spectrometer

(Applied Biosystems). We found that this protein corresponded to PEBP-1 from *Bos Taurus* (unused score: 14.92, coverage of the sequence: 54.5% according to Protein Pilot software) and to PEBP-1 from *Homo sapiens* (unused score: 12.70, coverage: 37.4%).

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[**] N. Hori, C. Keon-Sang, K. Murakawa, R. Matoba, A. Fukushima, K. Okubo, K. Matsubara, A human cDNA sequence homologue of bovine phosphatidylethanolamine-binding protein, *Gene* 140 (1994) 293-294.

[***] B. Haendler, R. Hofer-Warbinek, E. Hofer, Complementary DNA for human T-cell cyclophilin, *EMBO J.* 6 (1987) 947-950.

[****] P.C. Simister, M.J. Banfield, R.L. Brady, The crystal structure of PEBP-2, a homologue of the PEBP/RKIP family, *Acta Crystallogr. D* 58 (2002) 1077-1080.

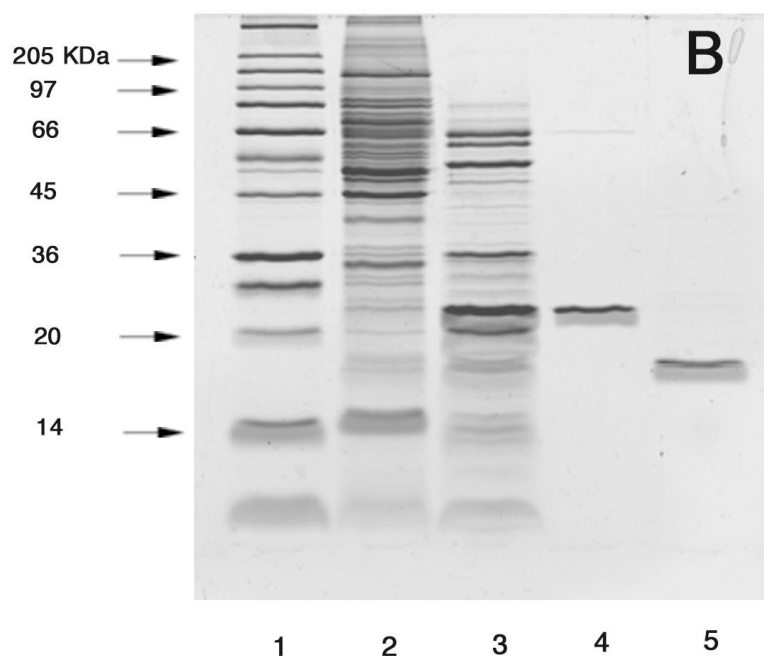
Supplementary file 2: Purification of Cyp A and PEBP from brain tissue

Purification of PEBP by hydrophobic interaction chromatography.

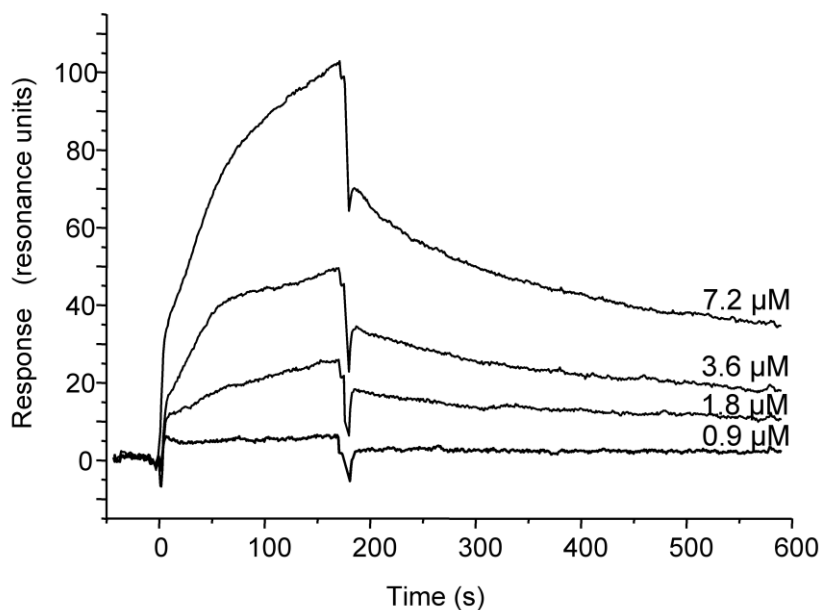
A 25 ml column (1.6x12 cm) of Octyl Sepharose 4B (GE Healthcare Bio-Sciences) was prepared. It was equilibrated against 50 per cent of buffer A (20 mM potassium phosphate, pH 7.5, 1mM EGTA, 1 mM MgSO₄) and 50 per cent of buffer B (3 M KCl in buffer A). Thirty ml of fraction R were equilibrated against a v/v mixture of buffer A and buffer B and poured on the column. The column was rinsed with 100 ml of the same buffer mixture, then, PEBP was eluted by decreasing linearly the concentration of buffer B from 50 to 0%. PEBP was eluted with about 35% of buffer B.

Purification of Cyp A by ion exchange chromatography.

A 50 ml column (1.6x25 cm) of CM sepharose (GE Healthcare Bio-Sciences) was equilibrated against 50 mM sodium acetate buffer pH 5.5, 1mM EGTA, 1mM MgSO₄ (buffer C). Fraction R (30 ml) was dialysed for one night against this buffer and loaded on the column. The column was rinsed with 100 ml of buffer C, then, a linear (200 ml) gradient from 0 to 50% of buffer D (50 mM sodium acetate pH 5.5, 1M KCl, 1mM EGTA, 1 mM MgSO₄) was applied. Cyp A was eluted with about 0.25 M KCl.

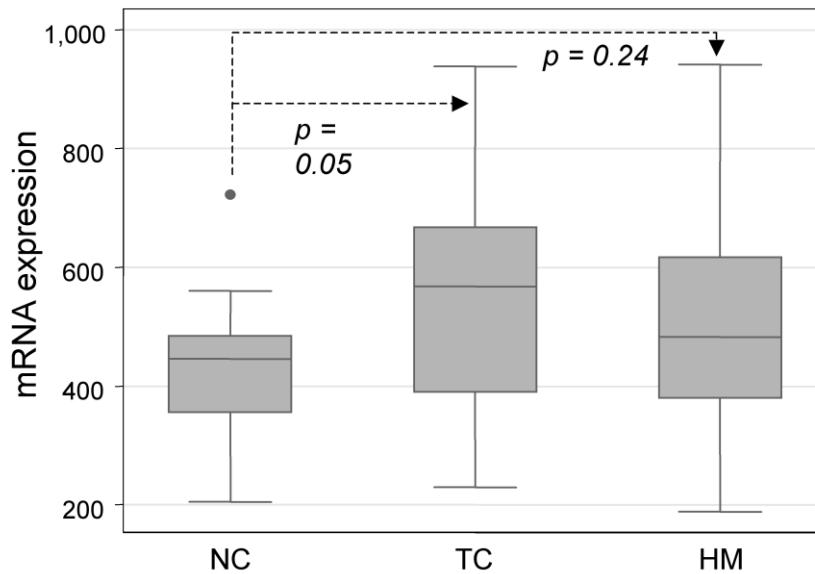


Electrophoretic patterns of purified samples of Cyp A and PEBP. A 15% polyacrylamide gel is run in denaturing conditions, it is loaded with molecular weight markers (lane 1), 40 μ g of brain cell supernatant (lane 2), 15 μ g of fraction R (lane 3), 3 μ g of purified PEBP (lane 4) and 3 μ g of purified Cyp A (lane 5). The gel is stained with Coomassie brilliant blue R250.

Supplementary file 3 : Surface plasmon resonance analysis.

The kinetic parameters of the binding of Cyt c on Cyp A was determined at 25 °C by surface plasmon resonance analysis using a Biacore 3000 instrument (GE Healthcare, Biacore AB Uppsala, Sweden). Cyp A was covalently immobilized in 10 mM sodium acetate buffer pH 4.0, on a CM5 sensor chip surface, using a standard amine coupling method. A level of 7500 resonance units (RU) of Cyp A was obtained. A control flow cell was prepared in the same conditions without protein. Different concentrations (0.9-7.2 μM) of Cyt c in HBS buffer (10mM HEPES pH 7.4, 3 mM EDTA, 0.005 % non-ionic surfactant P20, 300 mM NaCl) were injected at a flow rate of 30 $\mu\text{l}/\text{min}$. The dissociation phase was followed by a regeneration step with 1M NaCl. All the sensorgrams were corrected by subtracting the control flow cell response. The data were globally fitted to the 1.1 Langmuir model using Biaevaluation 4.1 software.

Supplementary file 4: Cyp A mRNA expression in tissue samples of 27 patients:



The tissues samples belong to a set of clinical specimen already described [*]. Tumor and non-tumor colon samples have been collected following a standardized procedure in order to obtain high quality RNA [**]. First strand cDNA synthesis was performed using a T7-linked oligo-dT primer and was followed by second strand synthesis. Labeled cRNA probes were then generated by reverse transcription followed by *in vitro* transcription, incorporating biotin labels, as part of the standard Affymetrix protocol. For each sample, the probes were then hybridized to human genome U133 chips (Affymetrix Inc., Santa Clara, CA), corresponding to genes and expressed sequence tags. Probes were then scanned, and signal intensity for each transcript was determined using MAS 5.0 Software (Affymetrix Inc.). Inter-array normalization was performed using a set of internal standard genes for the determination of a scaling factor.

The figure represents the Box plot of Cyp A mRNA levels determined by Affymetrix technology; NC: normal colon (18 samples), TC: tumor colon (20 samples), HM: hepatic metastasis (19 samples). The box for each tissue represents the interquartile range (25 to 75th percentile) and the line within this box is the median value. Bottom and top bars of the whisker indicate the 10th and 90th percentile respectively. Outlier values are indicated (closed circle). The comparison of Cyp A mRNA expression between the different tissues was done using the Wilcoxon exact test, the *p*-values are indicated on the figure.

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