RESEARCH COMMUNICATION

Direct demonstration of a specific interaction between cyclophilin-D and the adenine nucleotide translocase confirms their role in the mitochondrial permeability transition

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A fusion protein between cyclophilin-D (CyP-D) and glutathione S-transferase (GST) was shown to bind to purified liver inner mitochondrial membranes (IMMs) in a cyclosporin A (CsA)-sensitive manner. Binding was enhanced by diamide treatment of the IMMs. Immobilized GST–CyP-D avidly bound a single 30 kDa protein present in Triton X-100-solubilized IMMs; immunoblotting showed this to be the adenine nucleotide translocase (ANT). Binding was prevented by pretreatment of the CyP-D with CsA, but not with cyclosporin H. Purified ANT also bound specifically to GST–CyP-D, but porin did not, even in the presence of ANT.

INTRODUCTION

Mitochondria possess a latent non-specific pore within their inner membrane whose activation is thought to be a critical event in the induction of both necrotic and apoptotic cell death [1–3]. Often known as the mitochondrial permeability transition pore (MPTP), its molecular identity has not been firmly established. We proposed in 1990 [4] that the MPTP is formed by mitochondrial cyclophilin (CyP)-D binding to the adenine nucleotide translocase (ANT), followed by a conformational change in the ANT that is triggered by matrix Ca^{2+}. Since that time we and others have provided a substantial body of circumstantial evidence to support this model. We demonstrated that mitochondria contain a unique, nuclear-encoded cyclophilin, CyP-D, whose affinity for cyclosporin A (CsA) and its analogues closely matches their ability to inhibit the MPTP [1,5,6]. Furthermore, we have shown that CyP-D binds to the inner mitochondrial membrane (IMM) in a CsA-sensitive manner, and this binding is increased under conditions, such as oxidative stress and treatment with diamide, that enhance the sensitivity of the MPTP to $[Ca^{2+}]$ [7–9]. We have also shown that inhibition of the MPT by ADP involves binding of the nucleotide to the ANT and that this binding is reduced by modification of specific thiols on the ANT [9]. Others have shown that the purified and reconstituted ANT can be converted into a non-specific pore at high (> 1 mM) Ca^{2+} but, unlike the MPTP, the resulting pore is not inhibited by CsA [10,11].

In order to confirm that the ANT within the IMM binds CyP-D we have overexpressed CyP-D as a fusion protein with glutathione S-transferase (GST). This enabled us to produce a CyP-D affinity matrix which we have employed to identify proteins present in Triton X-100-solubilized IMMs that bind in a CsA-sensitive manner. Here we report that a single 30 kDa protein binds specifically and this was confirmed to be the ANT by immunoblotting.

EXPERIMENTAL

Materials

Mitochondria and IMMs

Mitochondria were isolated from livers or hearts of 250 g male Wistar rats and purified by Percoll density-gradient centrifugation [4]. Mitochondria from *Saccharomyces cerevisiae* were prepared as described by Daum et al. [12]. When required, mitochondria were incubated at room temperature for 5 min in sucrose isolation buffer containing either 1 mM diamide or 1 mM dithiothrietol (controls) before preparation of IMMs using digitonin and Lubrol [13].

GST–CyP-D fusion protein

cDNA for rat CyP-D [6] was cleaved out of pBluescript using the restriction enzymes *Apa*1 and *Sma*1 and a suitable construct for splicing into the pGEX-4T-3 vector (Pharmacia Biotech), synthesized by PCR using a high-fidelity *Taq* polymerase. The integrity of the resulting GST–CyP-D insert was confirmed by automated sequencing. Over-expression of the fusion protein was achieved in DE3 cells following induction with isopropyl β-D-thiogalactoside and harvested cells were lysed by sonication in PBS, pH 7.3, containing 1 mM EGTA, 1 mM EDTA, 1 % Triton X-100, 1 mM 2-mercaptoethanol and protease inhibitors (1 μg/ml pepstatin, 1 μg/ml antipain, 1 μg/ml leupeptin and 1 mM PMSF). Purification was achieved in a single step using glutathione-Sepharose 4B (Pharmacia Biotech, St. Albans, U.K.) as described by the manufacturer. CyP-D was generated by throm-

Abbreviations used: ANT, adenine nucleotide translocase; CyP, cyclophilin; BKA, bongkrekate; GST, glutathione S-transferase; CAT, carboxyatractyloside; IMM, inner mitochondrial membrane; MPT, mitochondrial permeability transition; CsA, cyclosporin A; MPTP, mitochondrial permeability transition pore; CsH, cyclosporin H.

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bin cleavage of the GST–CyP-D and removal of GST using glutathione-Sepharose 4B.

Purified ANT

ANT was extracted from rat heart muscle mitochondria with 6% Triton X-100 and purified exactly as described previously [11].

Antibodies

Anti-porin antibodies were from Calbiochem (Nottingham, U.K.), whereas the other polyclonal antibodies were raised in rabbits using standard protocols described previously [14]. For anti-CyP-D antibodies, the over-expressed CyP-D was employed, whereas for rat ANT, antibodies were raised against the C-terminal 12 amino acids conjugated to keyhole limpet haemocyanin through an N-terminal cysteine. Antibodies against the ANT from *S. cerevisiae* were a gift from Dr Gerard Brandolin (Department of Biological and Molecular Structures, CNRS, Grenoble, France).

Methods

GST–CyP-D affinity column

GST–CyP-D was bound to glutathione-Sepharose 4B (2.5 mg of protein per ml of 50% slurry) and the Sepharose beads were collected by centrifugation and washed in KPi buffer [20 mM KH_2PO_4/0.1 mM EDTA/0.5% (w/v) Triton X-100, pH 7.2]. When the effects of CsA and cyclosporin H (CsH) were to be determined, the GST–CyP-D resin was incubated with 25 μM CsA/CsH before addition of IMMs to ensure total saturation of CyP with the drug. The IMMs were partially solubilized at 1 mg of protein per ml of KPi buffer containing 0.5% Triton X-100 and, where required, 0.1 μM CsA/CsH, 20 μM carboxyatractyloside (CAT) or 20 μM bongkrekate (BKA). Fractions (1 ml) of solubilized IMMs were incubated at 25 °C for 30 min with 50 μl of 50% GST–CyP-D-Sepharose and the resin was sedimented by centrifugation. After washing five times with 1 ml of KPi buffer the resin was resuspended in KPi buffer containing 3% Triton X-100 before three additional washes with KPi buffer, a wash with high-salt buffer (containing 500 mM NaCl) and then finally three further washes with KPi buffer. The GST–CyP-D and any bound IMM proteins were eluted from the resin by incubation with elution buffer (KPi containing 10 mM glutathione) at 25 °C for 30 min before analysis by SDS/PAGE and Western blotting. Samples of purified ANT (see below) were diluted 1 to 8 with KPi buffer before being applied to the affinity matrix and were subsequently treated in the same manner as the solubilized IMMs.

SDS/PAGE, silver staining and Western blotting

These were performed as described previously [7–9,13]

RESULTS AND DISCUSSION

The GST–CyP-D fusion protein (43 kDa) was successfully overexpressed; it could be readily purified on glutathione-Sepharose and cleaved using thrombin to produce CyP-D (19 kDa) as shown in Figure 1. The peptidylproline *cis–trans*-isomerase activity per nmole of both GST–CyP-D and the thrombin-cleaved CyP-D were similar to that of conventionally purified CyP-D [5] (results not shown), and in Western blots both reacted

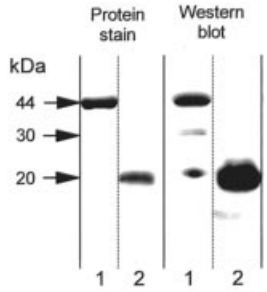

Figure 1 Purification and thrombin cleavage of overexpressed GST–CyP-D

The overexpressed protein was purified using glutathione-Sepharose and cleaved with thrombin as described in the Experimental section. SDS/PAGE was performed and the gel was analysed by Coomassie Blue staining (left-hand lanes) and Western blotting with anti-CyP-D antibodies (right-hand lanes). Lanes 1 and 2 show the GST–CyP-D before and after cleavage with thrombin and removal of GST on glutathione-Sepharose.

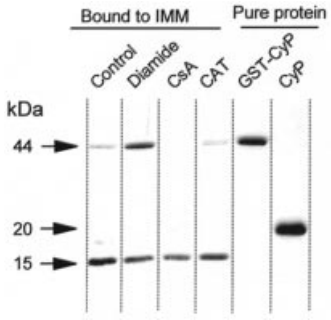

Figure 2 Diamide treatment increases and CsA inhibits GST–CyP-D binding toIMMs

IMMs (1.2 mg of protein) from diamide-treated or control mitochondria were incubated at room temperature for 10 min in a total volume of 150 μl of KPi buffer containing 15 μg of purified GST–CyP in the presence and absence of 20 μM CAT or 5 μM CsA as indicated. IMMs were sedimented by centrifugation and washed with 200 μl of KPi buffer before analysis by SDS/PAGE and Western blotting with anti-CyP antibodies.

with a polyclonal antibody raised to purified CyP-D (Figure 1). The GST–CyP-D was shown to bind to purified IMMs in a CsA-sensitive manner and this binding was increased when IMMs were prepared from mitochondria pre-treated with diamide (Figure 2). In four separate preparations, the amount of GST–CyP-D bound to IMMs from diamide-treated mitochondria was 298 ± 41% (mean $\pm$ S.E.M.) of that bound to controls. CyP-D itself, produced by thrombin cleavage, gave similar results (not shown). Treatment of the IMMs directly with diamide (as opposed to preparing the IMMs from diamide-treated mitochondria) did not lead to an increase in CyP binding. This is consistent with our previous conclusion that the diamide may require matrix glutathione to exert its effect [9]. It should be noted that the IMMs contain an endogenous protein of about 15 kDa that reacts with the anti-CyP-D antibody. This was not displaced by CsA but could represent endogenous CyP-D firmly bound to the IMMs [15,16] and proteolytically cleaved during the experimental protocol.

When IMMs were solubilized in 0.5% Triton X-100 (w/v) and then incubated with GST–CyP-D-Sepharose, a large number of proteins were bound (results not shown), but the great majority were removed by washing the matrix with buffers containing 3%

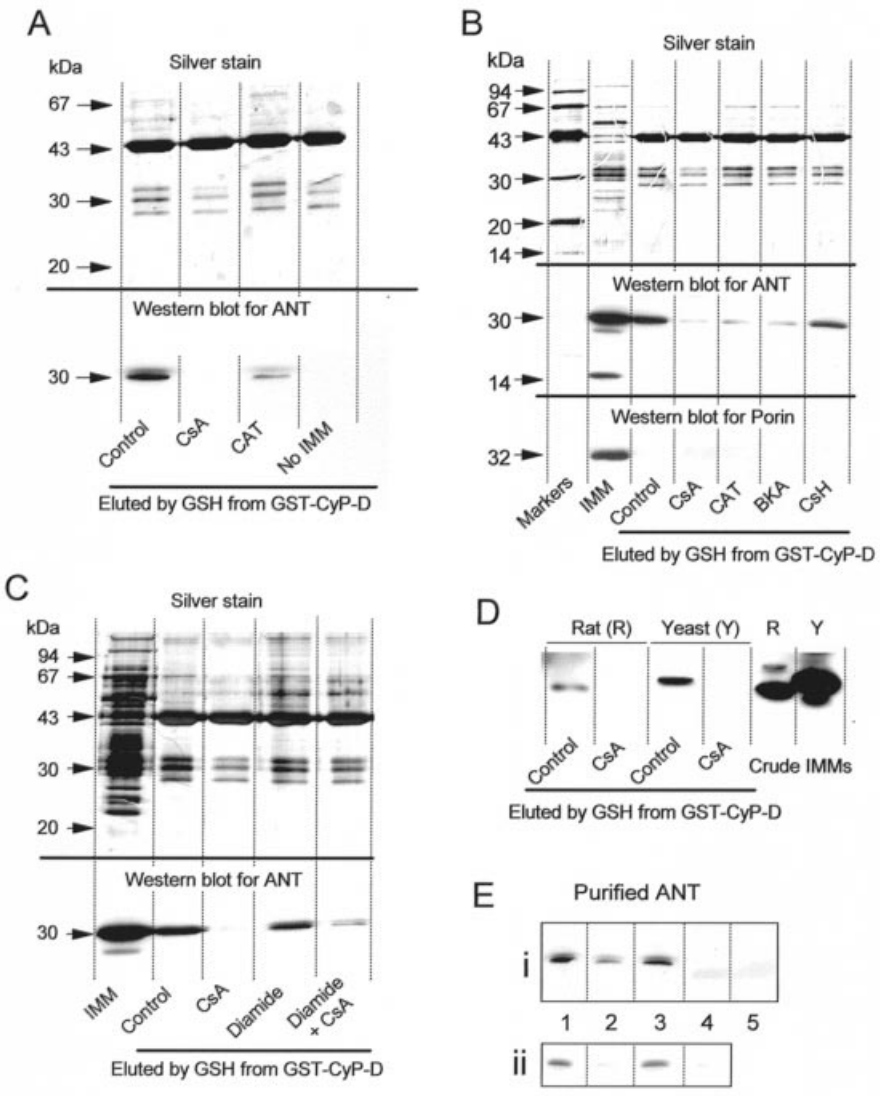

Figure 3 Binding of the ANT to a GST–CyP-D affinity resin

In (**A**) and (**B**) IMM proteins were preincubated with CsA (0.1 μM), CsH (0.1 μM), BKA (20 μM) or CAT (20 μM) as appropriate, and incubated with GST–CyP-D affinity resin as described in the Methods section. For incubations with CsA and CsH the resin had been pre-saturated with these drugs. After extensive washing of the resin (see the Methods section), bound proteins were eluted with glutathione and analysed by SDS/PAGE and Western blotting with anti-ANT or porin antibodies. In (**C**), results are shown for IMMs isolated from control and diamide-treated mitochondria, while in (**D**), binding of ANT present in IMMs prepared from rat liver mitochondria and yeast mitochondria are compared. Results in (**E**) were obtained using purified rat liver ANT. In (**E**) part i, lanes 1–3 show ANT binding to GST–CyP-D-Sepharose following incubation in the absence (lane 1) or presence of 25 μM CsA (lane 2) or 20 μM CAT (lane 3), whereas tracks 4 and 5 (with and without CsA respectively) show that no ANT was bound to GST-Sepharose. In (**E**) part ii, the purified ANT was treated with 1 mM dithiothreitol (lanes 1 and 2) or diamide (lanes 3 and 4) before incubating with GST–CyP-D affinity resin in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 25 μM CsA.

Triton X-100 and high salt concentrations (500 mM NaCl). The IMM proteins that remained bound, together with the GST–CyP-D, were eluted with glutathione, separated by SDS/PAGE and visualized using silver staining and Western blotting using anti-ANT antibodies. The results are shown in Figure 3. In Figure 3(A) the silver stain of the proteins eluted from the affinity matrix before addition of IMM (far right track) showed the presence of minor breakdown products of GST–CyP-D at 32 kDa, 30 kDa and 28 kDa in addition to the major protein band (43 kDa). We were unable to prevent this breakdown, despite the use of a range of protease inhibitors. After binding of solubilized IMM proteins, silver staining of the 30 kDa band was enhanced relative to the other bands, and this coincided with the detection of a band of 30 kDa in the ANT immunoblot. Pre-treatment of the GST–CyP-D affinity matrix with CsA, before addition of solubilized IMMs, prevented binding of the 30 kDa protein, and no ANT was detected in the Western blot. These results demonstrate that the ANT is bound very tightly to GST–CyP-D in a CsA-sensitive manner. However, once bound, the ANT could not be eluted from the GST–CyP-D using CsA (results not shown). This observation is consistent with the inability of CsA to displace CyP-D once it is bound to the inner membrane [7,8].

The interaction of the ANT with GST–CyP-D was greatly reduced if, before solubilization in Triton X-100, IMMs were treated with CAT or BKA, specific inhibitors of the ANT that trap the carrier in the 'C' or 'M' conformation respectively (Figures 3A and 3B). In three such experiments with different membrane preparations the CAT-treated samples bound only

$15 \pm 6\%$ of the ANT that bound to the control samples, whereas for BKA the values in two such experiments were 39% and 13%. These results suggest that some conformational change of the ANT may be occurring during binding to the CyP, which is prevented by locking the conformation of the ANT with the two inhibitors. Such a conformational change may greatly enhance the affinity of CyP-D-binding to the ANT, thus explaining the ability of CsA to prevent binding but not to reverse it once it has occurred. In contrast, ADP had no effect on binding (results not shown), in agreement with earlier results that showed that ADP does not compete for CyP binding in isolated mitochondria [9]. CsH, an analogue of CsA that does not block the MPTP [17], was also unable to inhibit binding of the ANT to the GST–CyP-D (Figure 3B). It has been suggested that the MPTP may involve an interaction of porin with the ANT [3,18,19]. However, we were unable to detect any porin bound to the GST–CyP-D affinity matrix, despite its presence in the IMMs (Figure 3B).

Since IMMs prepared from diamide-treated mitochondria bind more GST–CyP-D than control IMMs (Figure 2), we investigated whether the ANT in these IMMs binds more tightly to GST–CyP-D. No difference between control and diamide-treated samples was found in the amount of ANT bound (Figure 3C). However, in two separate experiments with diamide-treated samples, CsA inhibited ANT binding to the affinity matrix by only 85% and 79% compared with $93 \pm 1\%$ ($n = 7$) in control samples. It is possible that the binding of CyP-D to the diamide-modified ANT is tighter than to control ANT, reducing the effectiveness of CsA to compete with the ANT for binding.

In Figure 3(D) we demonstrate that the ANT from solubilized IMMs prepared from yeast (*S. cerevisiae*) mitochondria also binds to the GST–CyP-D affinity matrix in a CsA-sensitive manner. This is significant because none of the three ANT isoforms of yeast mitochondria possess the proline residue (Pro-61) that we originally proposed [4,9] might be responsible for the binding of CyP to the ANT. Thus the site of interaction of CyP-D with the ANT remains to be determined.

It could be argued that the ANT binds to CyP-D through another protein that we are not detecting. To exclude this possibility we have purified the ANT from heart IMMs using hydroxyapatite chromatography and confirmed that this also bound to the GST–CyP-D affinity matrix in a CsA-sensitive, CsH-insensitive manner (Figure 3E, part i). Diamide treatment of the purified ANT neither increased binding nor reduced the ability of CsA to prevent binding (Figure 3E, part ii), which again is consistent with the involvement of matrix components such as glutathione in producing the effects of this reagent on CyP-D–ANT interactions. The inability of CAT to prevent binding of the purified ANT (Figure 3E, part i, lane 3) contrasts with its effects when added to membranes before solubilization. It is probable that this reflects the inability of the detergent-solubilized ANT to bind CAT [20].

CONCLUSIONS

The results we have presented confirm that the ANT can bind specifically to CyP-D, which is required for our model of the MPTP. It has already been shown that the purified ANT can form a non-specific pore when reconstituted into proteoliposomes and exposed to high concentrations (1 mM) of Ca^{2+} [10,11]. Since CyP-D is a peptidylproline *cis–trans* isomerase, it can be envisaged that its binding enables a calcium-induced conformational change around a proline peptide bond to occur at much lower [Ca^{2+}] than in its absence [7–9]. As discussed above, this is unlikely to be Pro-61, since this is absent in yeast ANT isoforms which also bind to CyP-D. Another matrix-facing proline residue that is conserved in ANT isoforms from all organisms is Pro-28 [21]. Attempts are underway to establish whether this is a critical residue for CyP binding.

Our past results do not support an essential role for components other than the ANT and CyP-D in the formation of the MPTP. Thus, we have explained how the effects of all the known modulators of the MPTP, such as adenine nucleotides, oxidative stress, thiol reagents, membrane potential and pH can be accounted for by their interaction with the ANT and CyP-D [9]. However, it is possible that outer membrane proteins, such as porin and the benzodiazepine receptor that have been implicated by others [3,18,19], may play a role in regulating MPTP activity, although we have been unable to detect any effect of protoporphyrin IX or benzodiazepine derivatives on the opening of the MPTP in isolated liver mitochondria. Nor have we found any difference in the properties of the MPTP measured in isolated mitochondria devoid of their outer membrane [9]. Nevertheless, there is evidence to suggest that the ANT and porin associate at the contact sites between the outer and inner membrane [19,22] and thus there may be some communication between the two systems. Whether this may provide a link between the opening of the MPTP and the release of cytochrome *c* from the intermembrane space that occurs early in apoptosis remains to be established [23].

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