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DEMONSTRATION OF TERNARY IMMUNOPHILIN–CALCINEURIN COMPLEXES WITH THE IMMUNOSUPPRESSANTS CYCLOSPORIN AND MACROLIDE FK506

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Abstract—The specificity of cyclosporin A (CsA) binding to the major intracellular receptor proteins, cyclophilin A and B, as well as the interaction of CsA with the phosphatase calcineurin were investigated. Binding of photoaffinity-labeled CsA (PL-CS), a photoaffinity probe of CsA, to recombinant human cyclophilin A and B is saturable and specific. Non-specific PL-CS binding to calcineurin is observed in the absence of cyclophilin and calmodulin. In the presence of cyclophilin, cyclosporin–calcineurin binding becomes specific. Ternary complexes containing an equimolar ratio of cyclophilin A or B, PL-CS and calcineurin are resolved using the chemical-crosslinking technique. The formation of these complexes is specific, calcium- but not calmodulin-dependent, and is only inhibitable by cyclosporins, which bind cyclophilin. The drug–immunophilin complex binds to the calcineurin A subunit. The proteolytic 43 kDa product of calcineurin A retains binding properties, suggesting that the C-terminal domains are not necessary for complex formation. A trimeric complex of FKBP–calcineurin is also formed with FK506, but not with rapamycin. As expected, these complexes are only competed with by homologous derivatives. Chemical crosslinking of photolabeled Jurkat T-cells strongly suggests that drug–calcineurin complexes are of biological relevance.

Key words: photoaffinity labelling; calcineurin; immunophilin; Jurkat cells

The immunosuppressants CsA†, FK506 and the structurally related macrolide, rapamycin, are interesting tools for investigating T-cell activation [1–3]. These two types of drug bind to distinct cellular receptors, collectively termed immunophilins [4]: cyclophilins for cyclosporin [5–9] and FKBPs for the macrolides FK506 and rapamycin [9–12]. Although FK506 and CsA bind to distinct cellular receptor proteins, they have essentially identical effects on T-cell inactivation [13–15]. These findings suggest that the two immunosuppressants interfere with a common pathway of T-cell activation. The recent demonstration of a common target, i.e. calcineurin, was an important finding supporting this model [16, 17]. The experimental evidence was binding of the complex of calcineurin and calmodulin on cyclophilin- or FKBP-affinity matrices, which only occurred in the presence of CsA or FK506. Furthermore, both immunophilin–CsA and –FK506 complexes inhibited the protein phosphatase activity of bovine brain calcineurin. Additional evidence supporting an important role for activated calcineurin phosphatase in T-cell activation was recently reported [18–20]. A substrate of calcineurin involved in T-cell activation the transcription factor NFATp has been identified [21, 22].

Rapamycin, which binds to the same intracellular receptor, FKBP, as FK506, inhibits CsA- and FK506-resistant T-cell activation pathways, including IL-2-dependent signal transduction. Recent data indicated that IL-2 causes S6 kinase activation, which is a rapamycin-sensitive step [23, 24].

Calcineurin is a Ca^{2+}- and calmodulin-dependent serine-threonine protein phosphatase (2B-PP), consisting of two subunits with molecular masses of 59 and 17 kDa [25]. Calcineurin belongs to the family of neutral phosphatases, which include PPase-1 and 2A, and is expressed ubiquitously in eukaryotic cells, as well as in yeast [26].

The purpose of the present investigation was to characterize the nature and specificity of cyclosporin and FK506 binding to their cytosolic receptor proteins, the immunophilins, using a photoaffinity labeling technique with a cyclosporin derivative (PL-CS), and to demonstrate immunophilin–ligand complex binding to calcineurin by chemical crosslinking techniques. We show specific drug binding to the immunophilins and complex formation with calcineurin A. Furthermore, we were able to demonstrate the presence of ternary complexes of immunophilin, drug and calcineurin A in intact Jurkat T-cells.

MATERIALS AND METHODS

Materials. The photoaffinity labeling derivative of cyclosporin, PL-CS (SDZ 212-122), described previously [27, 28], CsA, CsH and 211-810 were obtained from Sandoz (Basel, Switzerland) and

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† Abbreviations: CsA, cyclosporin A; CsH, cyclosporin H; PL-CS, photoaffinity-labeled CsA; FKBP, FK506 binding protein; DST, disuccinimidyl-tartarate; HRP, horse radish peroxidase.

dissolved in ethanol at 10 mM. Antibodies to recombinant human cyclophilin A and B were raised in rabbits. Sheep antibodies against calmodulin were obtained from Polyscience (Warrington, PA, U.S.A.). Murine monoclonal antibodies to CsA (mab 45/45) were from Dr V. F. Quesniaux [29]; recombinant human cyclophilins A, B and FKBP, as well as calcineurin and calmodulin were obtained from Dr M. Zurini (Sandoz Pharma, Basel).

Photolabeling. Jurkat cells (5 Mio. cells) or purified proteins (0.1–10 μM) were incubated with 0.1–10 μM PL-CS in 24 or 96 well plates for 30 min at room temperature in the dark, and were then exposed to UV light (UV lamp, model B-1004, Blake-Ray, CA, U.S.A.) for 5 min on ice at a distance of 10 cm [30]. After lysis of cells, samples were mixed with gel sample buffer (20% glycerin, 2% SDS, 62.5 mM Tris–HCl, 0.04% bromophenol blue, pH 6.8 in distilled water) containing 5% 2-mercaptoethanol and heated at 95° for 3 min. Samples were subjected to electrophoresis on 15% (w/v) polyacrylamide gels according to Laemmli [31]. The gels were either silver stained or directly transferred to nitrocellulose membranes.

Chemical crosslinking. Proteins (1 mg cell lysates, cyclophilin and calcineurin at 1 μM in PBS) were incubated in a volume of 50 μL with 2 mM DST (Pierce, Rockford, IL, U.S.A.) for 15 min at room temperature. The reaction was stopped by adding gel sample buffer.

Western blotting. Western transfers were performed as described [27]. In brief, gels were presoaked in Tris (25 mM), glycine (150 mM) buffer at pH 8.3 containing 10% methanol for 15 min and proteins were transferred onto ECL-Hybond nitrocellulose membrane (Amersham, U.K.) by electrophoretic blotting at 0.1 V overnight (Trans-Blot Cell, Bio-Rad). After blocking with 5% milk powder, 0.25% Tween 20 in PBS the membrane was incubated with either murine anti-CsA mAB 45/45 [29] or rabbit anti-cyclophilin serum. Subsequently, the membranes were incubated with sheep anti-mouse immunoglobulin-HRP (Amersham) or donkey anti-rabbit immunoglobulin-HRP (Amersham). The immunoreactive proteins were developed with the ECL western blotting detection system (Amersham).

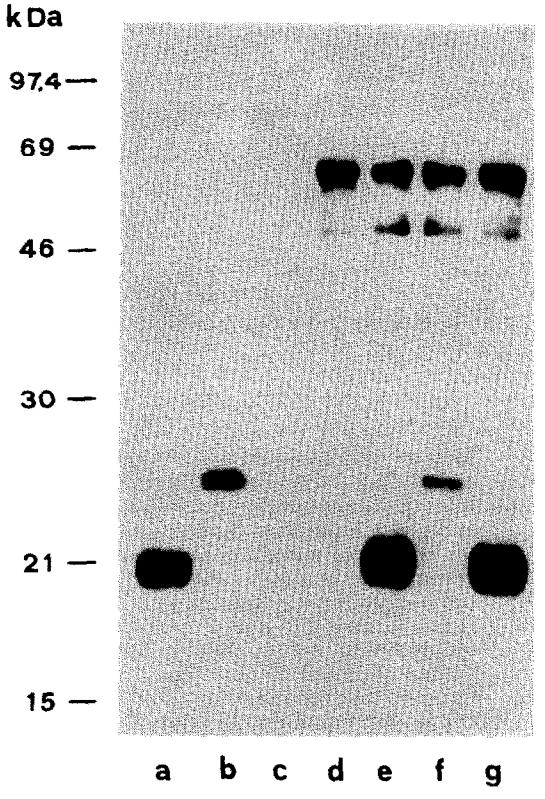

Fig. 1. Photoaffinity labeling of recombinant human cyclophilin A/B, purified bovine calmodulin and calcineurin with PL-CS. The proteins (1 μM) were incubated with 1 μM PL-CS, followed by UV light crosslinking, transfer on nitrocellulose membrane and detection with anti-CsA Mab. (a) Cyclophilin A, (b) cyclophilin B, (c) calmodulin, (d) calcineurin, (e) calcineurin and cyclophilin A, (f) calcineurin and cyclophilin B, (g) calcineurin, cyclophilin A and calmodulin.

RESULTS

Photoaffinity labeling of cyclophilin and calcineurin

For the direct identification of binding proteins we used a photoaffinity derivative of cyclosporin (PL-CS), which we have previously described and characterized in detail [27]. This derivative recognises both recombinant human cyclophilin A and B (Fig. 1a, b); binding of PL-CS is specific, e.g. is competed with by immunosuppressively active, but not inactive cyclosporin derivative [30], one interesting exception being 211-810, which is inactive although having high affinity for cyclophilin. Purified bovine calcineurin alone or in the presence of cyclophilin A or B binds PL-CS (Fig. 1d, e, f). The addition of bovine calmodulin does not change the photoaffinity labeling of cyclophilin and/or calcineurin (Fig. 1g). Under our experimental conditions calmodulin is not labeled (Fig. 1c). The results suggest that binding of PL-CS to calcineurin is independent of cyclophilin. Labeled proteins are visible at 48 and 60 kDa, the former being a known proteolytic degradation product of calcineurin A, while calcineurin B, which migrates at 16 kDa, is not labeled (Fig. 1d–g). PL-CS binding to calcineurin A is Ca^{2+} dependent and is abolished by the addition of the Ca^{2+} chelator EGTA (not shown).

Non-specific binding of cyclosporin to calcineurin

The binding of PL-CS to calcineurin in the presence of calcium was investigated in more detail. Binding of PL-CS to calcineurin A is detectable at 0.1 μM and increases linearly to 3 μM without reaching saturation (Fig. 2A). With PL-CS at 1 μM calcineurin A binding is detectable at calcineurin concentrations of 10–100 nM (Fig. 2B). The affinity of PL-CS is about 10 times less than that of cyclophilin A. In contrast to cyclophilin A, calcineurin A–PL-CS binding can be competed with by both active cyclosporin A and inactive cyclosporins, e.g. CsH and 211-810 (an inactive

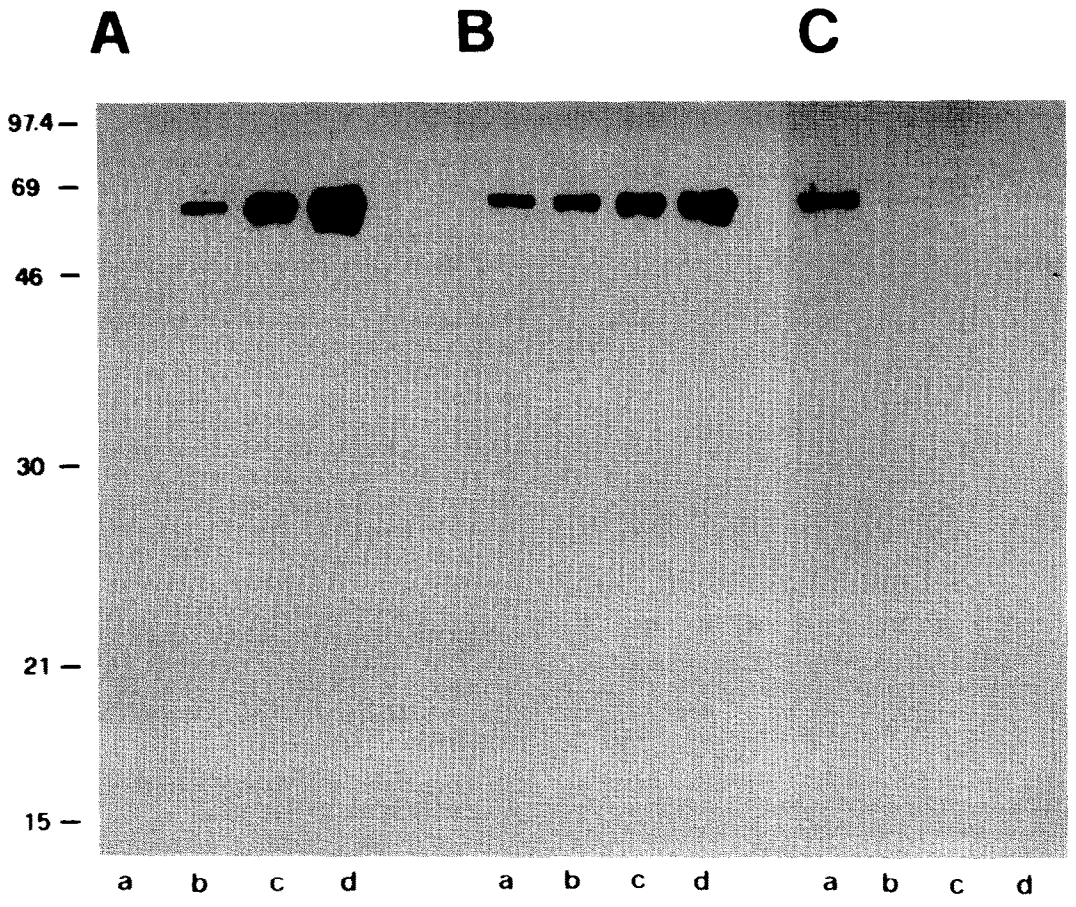

Fig. 2. Cyclosporin binding to calcineurin A is non-specific. Purified bovine calcineurin was incubated with PL-CS, photolabeled and detected by immunoblot using anti-CsA antibody. (A) Calcineurin (1 μ) was incubated with increasing concentrations of PL-CS (μM): (a) 0.1, (b) 0.3, (c) 1, (d) 3. (B) Increasing concentrations of calcineurin (μM) were incubated with 1 μM PL-CS: (a) 0.1, (b) 0.3, (c) 1, (d) 3. (C) Competition with PL-CS binding to calcineurin (each at 1 μM): (a) control, (b) CsA, (c) CsH, (d) 211-810, each at 3 μM.

derivative able to bind cyclophilin, Fig. 2C). PL-CS–calcineurin A binding in the absence of cyclophilin is therefore non-specific, e.g. does not distinguish between active and inactive cyclosporins, and is possibly due to hydrophobic interactions.

In the presence of cyclophilin cyclosporin–calcineurin binding becomes specific

In the presence of cyclophilin, photoaffinity labeling of calcineurin (Fig. 3a) is apparently specific, since it is inhibited by a 3-fold excess of CsA and 211-810 (Fig. 3b, d), but not CsH (Fig. 3c). Since PL-CS has only one reactive group, simultaneous incubation of cyclophilin and calcineurin with the photoaffinity probe engages either cyclophilin or calcineurin, and no ternary complexes are observed. Ternary complexes, however, may be formed and visualized by chemical crosslinking. The addition of the chemical crosslinker DST revealed a broader PL-CS-labeled protein at 95 kDa, in addition to cyclophilin and calcineurin (Fig. 3e). Labeling of this 95 kDa protein is again inhibited by CsA and 211-810, but not CsH (Fig. 3f–h). The labeled complex at 95 kDa is composed of several proteins, which were analysed further.

Site of cyclophilin–CsA binding to calcineurin

Limited proteolysis of calcineurin A resulted in a 43 kDa fragment, which retained full binding properties. This procedure removes the C-terminal 17 kDa of calcineurin A, which contains the inhibitory and calmodulin binding domains. These domains are therefore unlikely to be involved in binding (not shown).

Composition of calcineurin–cyclophilin complexes

Chemical crosslinking with DST of the purified proteins alone or in combination in the presence of PL-CS followed by silver staining reveals novel protein complexes at 80 and 95–100 kDa (Fig. 4A, lane d); in addition, DST induces a broad protein complex at 16–20 kDa, which occurs in the absence of CsA and is non-specific. Using the more sensitive immunoblot technique the higher molecular mass

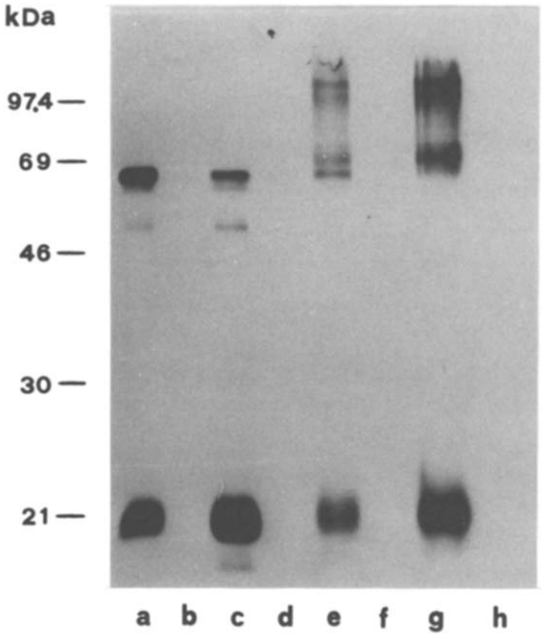

Fig. 3. Cyclophilin converts the CsA binding specificity of calcineurin. Direct evidence of ternary complex. Cyclophilin (1 μM) and calcineurin (3 μM) were photoaffinity labeled in the presence or absence of competitor. Competition with 1 μM PL-CS by solvent (a), CsA 10 μM (b), CsH 10 μM (c) and 211-810 10 μM (d). Chemical crosslinking with DST of photoaffinity-labeled cyclophilin and calcineurin (e). Competition with ternary complex by 10 μM CsA (f), CsH (g) or 211-810 (h).

complexes are composed of several proteins: firstly, the 95 kDa contains cyclophilin (Fig. 4B, arrows in lane d); secondly, the 80 and 95 kDa contain calcineurin, and additional calcineurin-reactive proteins are detected at 118 and 130 kDa (Fig. 4C). The cyclosporin binding property of these proteins was assessed by photoaffinity labeling; PL-CS-labeled protein complexes are found at 95, 118 and 130 kDa (Fig. 4D). These results suggest that one cyclophilin molecule associates with the complex of calcineurin A and B in the presence of active drug resulting in the complex observed at 95 kDa. The higher molecular mass PL-CS-labeled proteins might represent complexes containing dimers of calcineurin A and B linked with or without cyclophilin. Alternatively, calcineurin might associate with dimers of cyclophilin, as proteins at 35 kDa reacting with the anti-cyclophilin antibody are detectable (Fig. 4B, lane c), which form only in the presence of PL-CS. The protein at 65 kDa reacting with the cyclophilin antibody might represent a complex of cyclophilin and a proteolytic calcineurin A product at 43 kDa, which cross-reacts with the same antibody (Fig. 4B).

FKBP–drug calcineurin complexes

The possibility of complex formation of FKBP–FK506 or rapamycin with calcineurin was analysed similarly (Fig. 5). As the molecular mass of FKBP is 12 kDa, a complex of FKBP, FK506 and calcineurin A and B is expected at 90 kDa, which is indeed the case, as a broad band at about 90 kDa can be demonstrated by silver staining (Fig. 5A).

The sensitive immunoblot technique revealed the presence of FKBP (Fig. 5B, lane d) in the 90 kDa protein complex. Furthermore, the complex is only formed in the presence of FK506, and not in the presence of rapamycin (Fig. 5B, lane f). This is the first direct demonstration of a ternary complex formed by FKBP–FK506 and calcineurin, but not by rapamycin. The anti-FKBP antibody cross-reacts with minor impurities of FKBP (not detected by silver stain) at 18 and 26 kDa and has minimal cross-reactivity with the purified calcineurin A preparation. However, an anti-FKBP-reactive protein complex appears at 30 kDa upon chemical crosslinking (Fig. 5b and d). This 30 kDa complex is only formed in the presence of FK506, and not of rapamycin. The nature of this specific complex is unclear except for the fact that it contains FKBP. It may contain calcineurin B, but the anti-calcineurin reactivity at 30 kDa is not increased by FK506. The most likely explanation is that the 30 kDa complex is composed of FKBP dimers, which are only formed in the presence of FK506, and not of rapamycin. The 90 kDa complex also contains, as expected, calcineurin (Fig. 5C).

Demonstration of immunophilin–drug–calcineurin complexes in Jurkat cells

In order to confirm that similar complexes may occur *in vivo*, we incubated Jurkat cells with PL-CS and performed chemical crosslinking on the cell lysate. As shown in Fig. 6A protein complexes at 90 kDa and at even higher molecular masses are labeled with PL-CS; the 90 kDa complex contains calcineurin (Fig. 6B) and cyclophilin (not shown). The resolution of the higher molecular complex is uncertain.

DISCUSSION

We demonstrate specific cyclosporin binding to cyclophilin A using a photoaffinity probe. Specific binding to calcineurin only occurs in the presence of cyclophilins. CsA binding to calcineurin depends on calcium, and is apparently independent of calmodulin. Calcineurin A and its proteolytic degradation products are the only labeled species under the experimental conditions.

Since the photoaffinity probe of CsA has one reactive site, cyclophilin–drug–calcineurin complexes are only obtained by the addition of a chemical crosslinker. We used several chemical crosslinkers with DST resulting in the least degradation and denaturation. A major problem of this approach is the fact that the higher molecular mass complexes enter with difficulty into SDS gels and therefore migrate more slowly. However, we were able to demonstrate complexes at 95, 118 and 130 kDa, which bound CsA, with the 95 kDa protein complex containing cyclophilin. The complexes were only formed in the presence of active cyclosporins. The 95 kDa protein complex is probably composed of calcineurin A and B, cyclophilin and CsA.

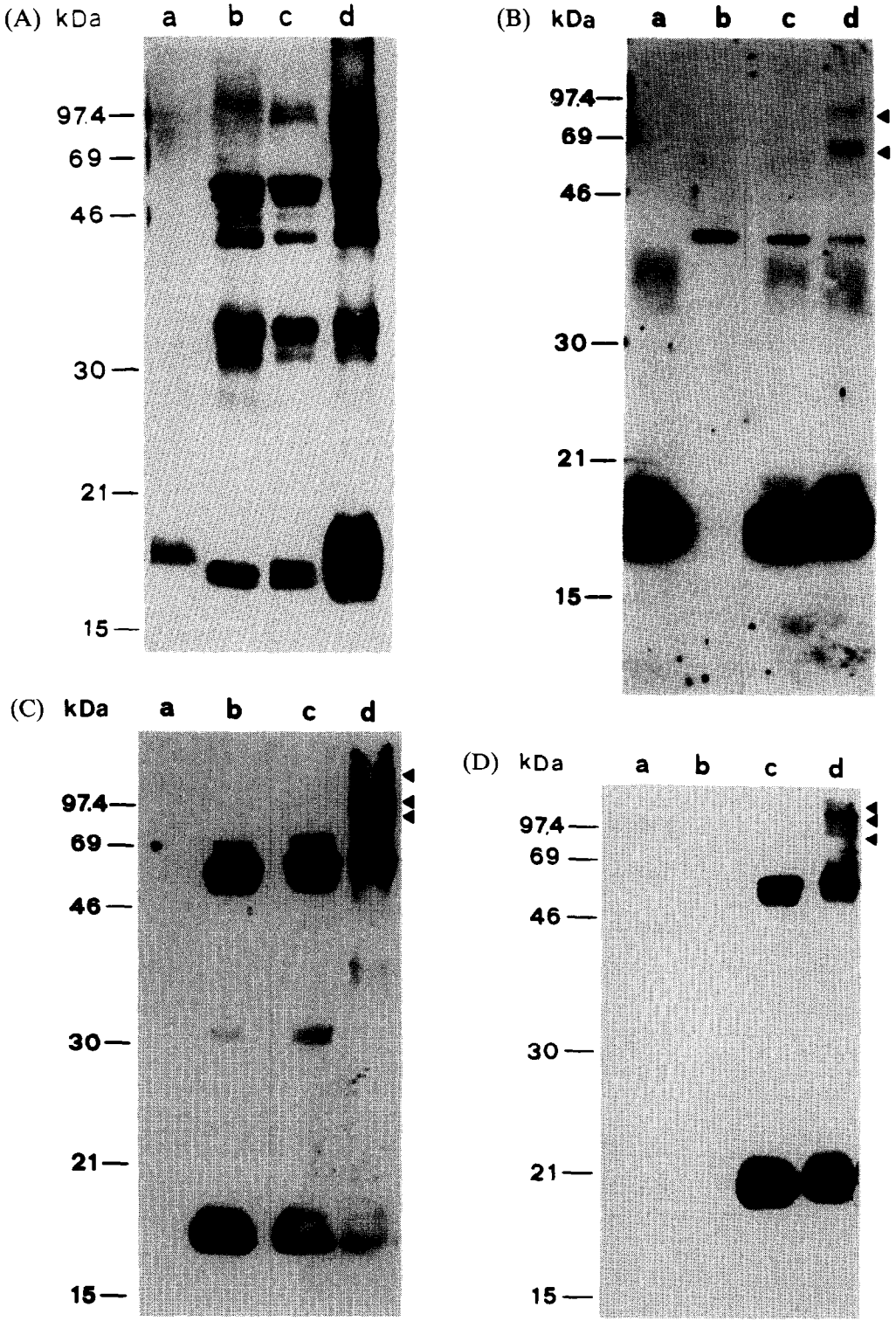

Fig. 4. Demonstration of the cyclophilin–CsA–calcineurin complexes. The proteins were incubated singly or in combination with photolabel and/or chemical crosslinker as indicated, separated on 15% SDS–PAGE and analysed by silver staining (A) or immunoblot (B–D). (A) SDS–PAGE silver staining: recombinant human cyclophilin (rhCyp) A (a), calcineurin (b), rhCypA, calcineurin and PL-CS at 1 μM (c), and rhCyp, calcineurin and PL-CS at 1 μM crosslinked with 2 mM DST (d). (B–D) Immunoblot analysis of the complex after chemical crosslinking with DST. Equimolar concentrations (1 μM) of rhCyp, PL-CS and purified calcineurin were crosslinked with DST, and the complexes analysed by immunoblot using anti-CypA antibody (B), anti-calcineurin antibody (C) or anti-CsA antibody (D). Groups as in (A).

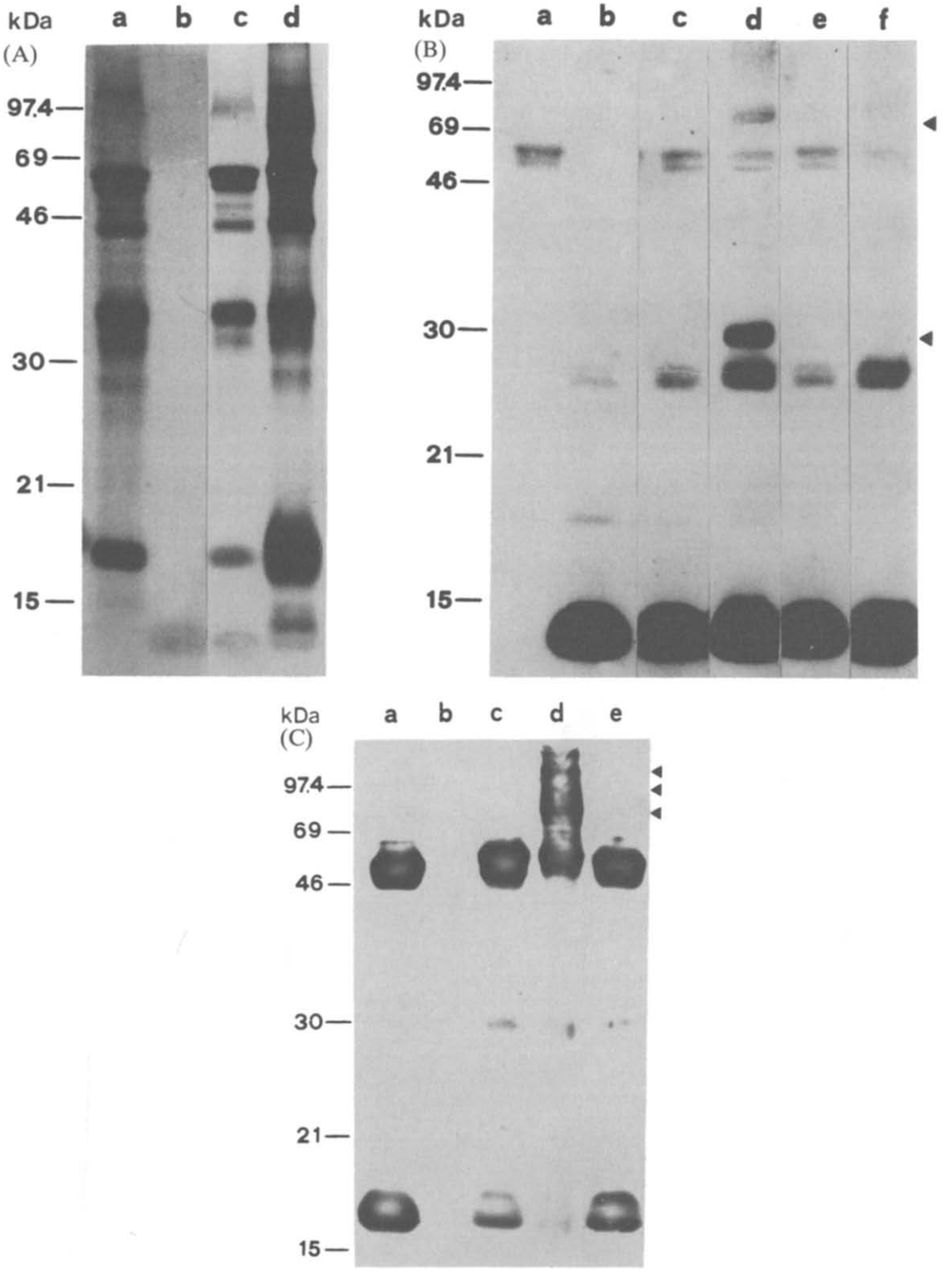

Fig. 5. Ternary complex formation of FKBP–FK506–calcineurin by chemical crosslinking (DST). The proteins were incubated singly or in combination with photolabel and/or chemical crosslinker as indicated, separated on 15% SDS–PAGE and analysed by silver staining (A) or immunoblot (B, C). (A) SDS–PAGE silver staining: calcineurin (a), recombinant human FKBP (rhFKBP) (b), rhFKBP, calcineurin and FK506 at 1 μM in the absence (c) or presence of 2 mM DST (d). (B) Immunoblot with anti-FKBP antibody: calcineurin (a), rhFKBP (b), rhFKBP, calcineurin and FK506 at 1 μM in the absence (c) or presence of 2 mM DST (d), rhFKBP, calcineurin and rapamycin at 1 μM in the absence (e) or presence of 2 mM DST (f). (C) Immunobolot analysis with anti-calcineurin antibody: calcineurin (a) and FKBP (b) were crosslinked with 2 mM DST in the absence (c) or presence of FK506 (d) or rapamycin (e), all reagents at 1 μM.

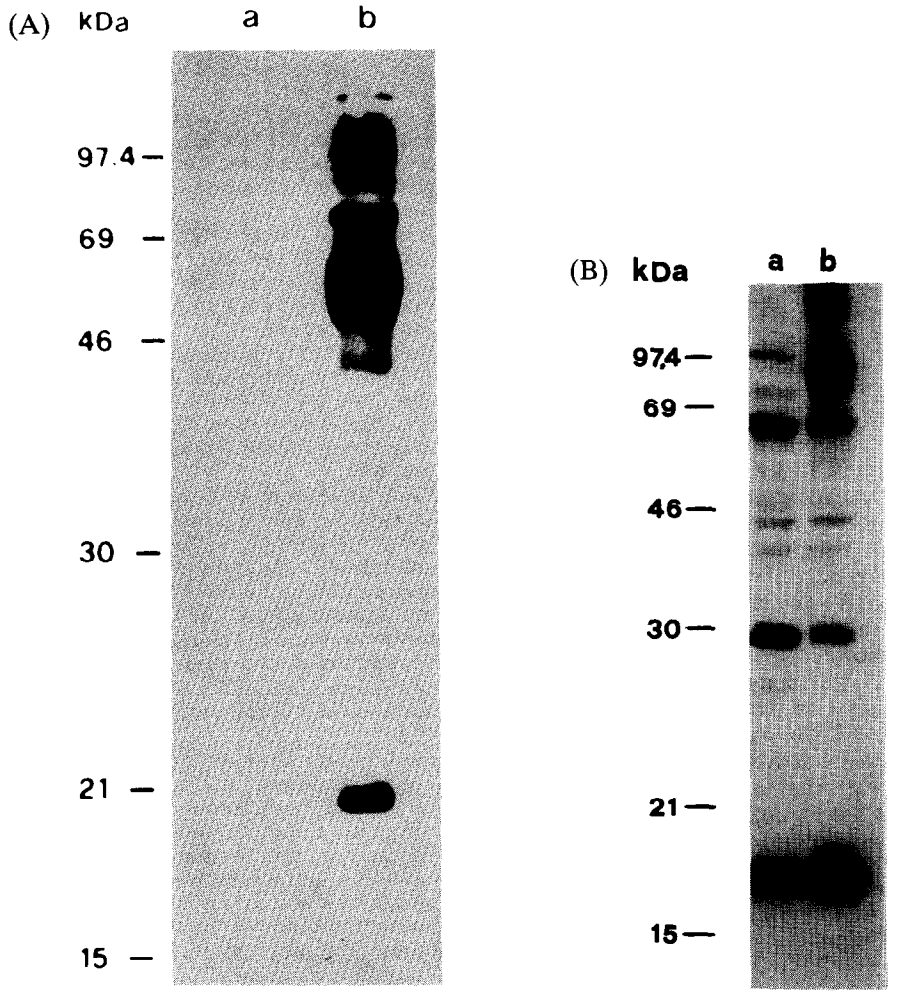

Fig. 6. Ternary complex in Jurkat T-cells. Cells (5×10^6) were incubated with 1 μM PL-CS, UV crosslinked, lysed and the lysate was crosslinked with DST; proteins were separated on a 15% SDS–PAGE, transferred on nitrocellulose and the labeled proteins detected with anti-CsA mAb (A) or anti-calcineurin antibodies (B). Cell lysate (1 mg protein) in the absence (a) or presence of 2 mM DST (b).

For the macrolide binding protein (FKBP) we show complexes consisting of FKBP and calcineurin A and B with FK506, but not with rapamycin or CsA. Complexes of FKBP with calcineurin in the presence of heterologous ligands could not be demonstrated [32].

The photoaffinity-labeled CsA derivative reveals additional CsA binding proteins at 40–45 and at 60 kDa in Jurkat T-lymphocytes [30]. The latter includes calcineurin A, while the former may be identical to actin [33], an as yet uncharacterized 40 kDa protein with certain homology to cyclophilin [34] and a 45 kDa phosphoprotein [35].

Calcineurin A and B and the proteolytic degradation products of the A subunit are easily detectable in Jurkat T-cells although at distinctly lower levels than those reported in brain tissue [18, 36]. Calcineurin A and its degradation product are photolabeled along with cyclophilin A. By chemical crosslinking of cell lysates higher molecular mass complexes containing CsA, cyclophilin and calcineurin can be resolved. These data suggest that ternary complexes or even complexes of higher order might occur *in vivo*. Together with the recent finding that calcineurin phosphatase in Jurkat T-cells is CsA sensitive [18], this provides compelling evidence for an important role of these proteins in the mechanism of CsA action.

While the inhibition of calcineurin activity by CsA depends on the presence of cyclophilin, the role of the individual cyclophilins in mediating inhibition *in vivo* is not yet clarified. *In vitro*, however, it was shown that cyclophilin B mediated a stronger inhibition of the calcineurin enzyme activity than cyclophilin A [37].

Although cyclophilin A and possibly also cyclophilin B have broad tissue distribution, that of cyclophilin C appears limited [38–40]. It has been speculated that the various members of this receptor family may mediate different functions, namely

inhibiting cytokine gene transcription versus toxicity [42]. Cacalano *et al.* [43] reported a specific membrane receptor for cyclosporin, which is not yet molecularly defined, and the possible relation to cyclophilin is unknown. Several membrane transporter proteins including the gp170 multi-drug resistance transporter [44, 45], the Na^+–D-glucose cotransporter in the kidney [46] and the bile transporter in the liver [47] have been identified as CsA target proteins (reviewed in Ref. 48). The relative importance of these proteins versus cyclophilin–calcineurin in inducing non-specific immunosuppressant effects, e.g. nephrotoxicity are presently under investigation.

With respect to the sites of the CsA molecule recognising cyclophilin, the studies of Quesniaux *et al.* [49] showed that the amino acids 11 and 1–3 are involved in binding to cyclophilin A. Thus, the amino acids on the opposite side of the ring structure of CsA are probably involved in binding to the calcineurin A molecule. Dual recognition for FK506 had also been suggested [50]. Whether the binding sites for the CsA–cyclophilin or FK506–FKBP complex on the calcineurin A molecule are identical is presently unknown.

In summary, we demonstrate that ternary complexes consisting of immunophilin, calcineurin and CsA and FK506 are only formed with immunosuppressively active derivatives. That such a chemically constructed complex is also demonstrable in Jurkat T-cells suggests that the formation of this ternary complex is of physiological relevance.

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