Controlling programmed cell death with a cyclophilin–cyclosporin-based chemical inducer of dimerization

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Background: Cell death can occur either from physical damage (necrosis) or cellular suicide (apoptosis). Apoptosis is essential for the development of multicellular organisms and disregulated apoptosis underlies many human diseases. The Fas receptor (Fas) is a membrane signaling protein that mediates a death signal following its aggregation by the Fas ligand. We have described methods to induce the association of proteins using cell-permeable molecules called chemical inducers of dimerization (CIDs). Here we describe the synthesis of a novel CID, $(CsA)_2$, that has two identical protein-binding surfaces derived from the immunosuppressant cyclosporin A (CsA). We use this CID to deliver a death signal to cells expressing a fusion protein containing cyclophilin (CyP, the protein receptor for cyclosporin) and the cytoplasmic signaling domain of Fas.

Results: $(CsA)_2$ was synthesized in six synthetic steps and 30 % overall yield from cyclosporin. It binds to two CyP proteins simultaneously, but does not inhibit T-cell signaling, presumably because the $(CsA)_2$–CyP complex does not bind to calcineurin. Jurkat cells stably transfected with constructs encoding myristoylated CyP–Fas fusion proteins undergo apoptosis in response to nanomolar quantities of $(CsA)_2$. Constructs containing a mutation in the myristoylation signal are defective for signaling.

Conclusions: The Fas signaling pathway can be activated with a cell-permeable CID derived from CsA in cells expressing an appropriately engineered Fas construct, which must be localized at the membrane. This new class of homodimerizing CIDs will be useful for in-depth analysis of protein association events in complex systems, including transgenic animals. Now that several CIDs with distinct dimerization characteristics are available, it should be possible to induce the activation of multiple pathways with complete specificity.

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Introduction

Cell death can occur by one of two processes, termed necrosis and apoptosis. In necrosis, physical damage to a cell causes a loss of membrane integrity, and death is usually accompanied by osmotic swelling and bursting of the cell. Apoptosis or programmed cell death (PCD) occurs primarily in multicellular organisms and is an active process in which a cell commits suicide for the benefit of the whole organism. This mode of cell death is recognized by characteristic morphological features including cytoplasmic and nuclear shrinkage, internucleosomal fragmentation (genomic DNA is fragmented at ~180 bp intervals), degradation of cellular components, and a loss of membrane integrity [1]. Several components of the apoptotic machinery have been identified, originally through genetic studies in nematodes [2]. Apoptosis mediated by the Fas receptor (Fas) has received special attention because of its exquisite specificity and its role in lymphoproliferative disorders [3]. Fas [4] (also known as APO-1 [5] or CD-95) is a type I transmembrane protein belonging to the tumor necrosis factor receptor (TNFR) superfamily of proteins (the name tumor necrosis factor is a misnomer, however, since this receptor causes cell death by apoptosis rather than by necrosis). Within this family, Fas and TNFR can cause apoptosis when engaged by their respective ligands. These proteins have homologous sequences within their intracellular domains, termed death domains, that contain the information necessary to trigger the apoptotic signal. Recently, several proteins that interact with Fas and/or TNFR have been identified [6–10]. Two of these, MORT1/FADD and TRADD can associate with each other as well as with Fas and TNFR, respectively, and thereby connect Fas and TNFR to their common downstream effector MACH1/FLICE [11–13]. MACH1/FLICE is a cysteine protease that is homologous to the ICE/CED-3 family of proteases and is activated upon signaling by Fas. Although the precise mechanism of activation of MACH1/FLICE by Fas and TNFR remains obscure, the likelihood that these receptors are activated

by aggregation led us to apply the chemical inducer of dimerization (CID) concept to this signaling pathway.

Physical contact between two or more molecules is required to relay information in signal transduction. Two outcomes to this interaction are possible; either the interaction results in a conformational change that facilitates the transfer of information (allostery), or it results in the localization of a molecule to the vicinity of an interacting partner or substrate (proximity) [14]. We previously described a technique for regulating the proximity of proteins with synthetic, cell-permeable molecules termed CIDs [15]. In the first illustration of this concept, a dimeric version of the naturally occurring ligand FK506 was synthesized, creating FK1012, a molecule with two protein-binding surfaces. A human T-cell line was transfected with a plasmid encoding a chimeric protein composed of three copies of human FKBP12 (the high affinity protein receptor for FK506 and rapamycin) [16,17] fused to ζ, a signaling component of the multisubunit T cell receptor (TCR). The addition of FK1012 to cells expressing this fusion protein aggregates the ζ fusion, thereby activating the endogenous TCR signaling pathway [15,18].

Since this original demonstration, FK1012 has been used to dimerize membrane receptors that activate the platelet-derived growth factor (Jianxin Yang, Mark Mercola and S.L.S. unpublished data) and Fas [19] pathways, and to recruit cytosolic signaling proteins that activate the Ras/MAPK [20] and Src [21] pathways. FK1012 can also signal in the nucleus, where transcription of a reporter gene is induced following the FK1012-mediated reassembly of two separated domains of a transcription factor [22]. Notably, FK1012 has also been shown to activate signaling in transgenic mice, demonstrating the potential for CIDs to be used to investigate signaling *in vivo* [19].

To expand this approach, we sought an additional receptor–ligand pair that could be modified into a dimerization domain and CID. Ideally, receptor–CID complexes should have high affinity, the CID should be cell permeable, the receptor should be monomeric, and neither the receptor nor the CID should have discernible effects on cells alone. The monomeric, 18-kDa cyclophilin (CyP) receptor and its cell-permeable ligand cyclosporin (CsA) bind with high affinity (K_d = 5 nM) [23] and thus appeared to be a promising system for development [24]. We have also created new receptor–ligand pairs based on CyP–CsA [23] by first modifying a residue of CsA such that it can no longer bind wild-type CyP. We then introduced compensatory mutations in the binding site of CyP such that the new CyP mutants gained the ability to bind the modified CsA. In this way, many such independent, non cross-reacting receptor–ligand pairs may be generated, each with the potential to be developed as a CID.

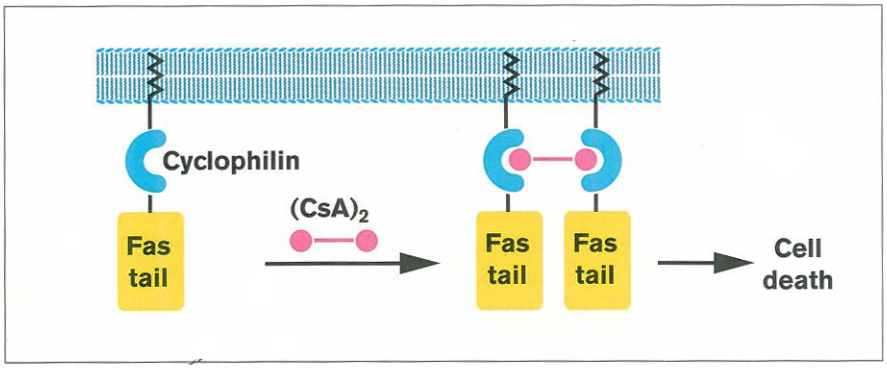
Like FK506, CsA's immunosuppressive and toxic properties are due to its ability to form an immunophilin complex that inhibits the protein phosphatase calcineurin [25]. When FK506 was converted into FK1012, the dimeric molecule was designed to be unable to form the complex surface that binds to calcineurin. Similarly, the conversion of CsA into its CID form required the rational removal of calcineurin-inhibitory properties. We now describe the synthesis of the cell-permeable CID $(CsA)_2$, and its use as an inducer of cell death in cells expressing a CID-responsive Fas receptor (Fig. 1).

Results

Synthesis of a CID derived from CsA

We used the known conversion of CsA to MeBmt-η-OH-1-CsA (**1**) [26] to install a functional group at the terminus of MeBmt-1 through a selective free radical bromination. The primary hydroxyl of the dihydroxy intermediate (**1**, Fig. 2) was converted to an activated carbamate with carbonyl diimidazole, providing an intermediate (**2**) that can be used to generate monomers, homodimers, or heterodimers. Reaction of this intermediate with xylylenediamine provided $(CsA)_2$. Reaction with benzylamine provided monomeric CsA-M (Fig. 2).

Figure 1



CID-inducible activation of a cellular signaling pathway. A chimeric protein composed of cyclophilin fused to the cytoplasmic tail of the Fas receptor and localized to the membrane by a myristoyl group can be caused to aggregate by the addition of the CID $(CsA)_2$. When the Fas tail is aggregated, the normal Fas signaling pathway is activated, leading to cell death.

In vitro demonstration of 2:1 binding stoichiometry

To determine whether $(CsA)_2$ has the ability to bind two molar equivalents of CyP simultaneously, we performed titration binding assays for CsA-M and $(CsA)_2$ with recombinant CyP. The assay relies on an approximately two-fold increase in CyP's fluorescence upon binding CsA [23,27]. The results show that $(CsA)_2$ can bind two molar equivalents of CyP whereas CsA-M can bind only one, as expected (Fig. 3).

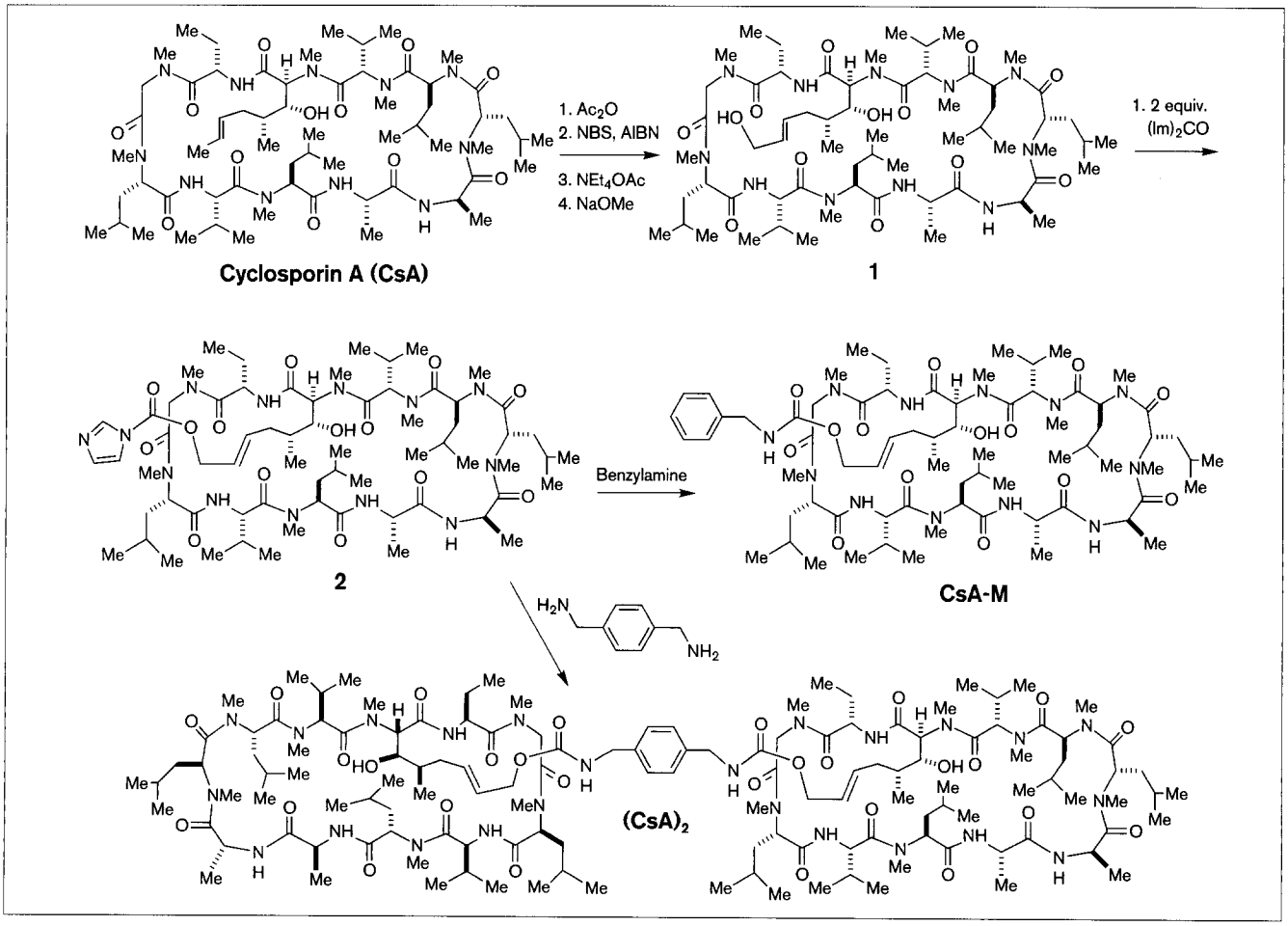
Assessment of $(CsA)_2$ and CsA-M inhibition of TCR signaling

The immunosuppressive properties of cyclosporin result from its ability to inhibit calcineurin when complexed with cyclophilin [25]. Calcineurin is a component of the TCR signaling pathway and is essential for induction of the transcription factor NFAT [28]. We used a cellular NFAT-signaling assay in which Jurkat T cells have been stably transfected with an NFAT-responsive β-galactosidase (β-gal) reporter gene [29]. Stimulation of these cells with ionomycin and phorbol myristate acetate (PMA) mimics activation of the TCR, induces NFAT, and causes the production of β-gal. As seen in Figure 4, CsA potently inhibits the production of β-gal activity, CsA-M has significantly reduced inhibition, and $(CsA)_2$ shows no inhibitory effects, even at 10 μM.

Regulation of Fas-mediated apoptosis with $(CsA)_2$

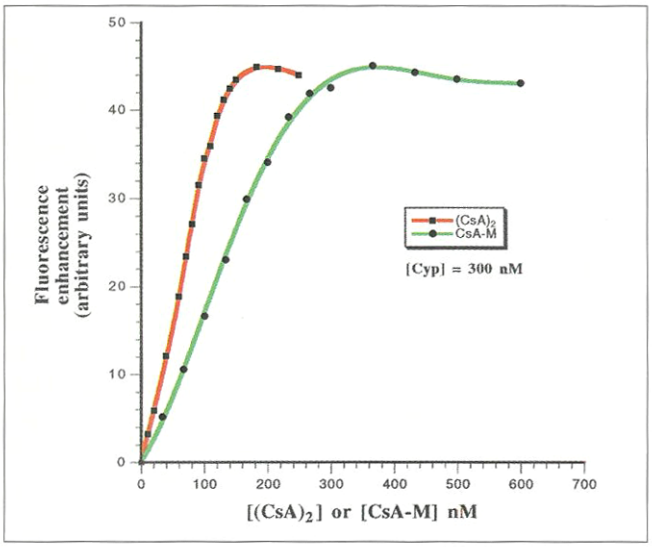
We prepared a series of DNA constructs encoding fusion proteins composed of (from N to C) a myristoylation-targeting peptide (for membrane attachment), from one to three CyP domains in tandem, the intracellular domain of Fas, and an epitope tag. Constructs having a reverse order of Fas and the CyP domains, and control constructs lacking the Fas domain or containing a non-functional myristoylation signal [30] were also prepared (Fig. 5). These were transiently transfected into Jurkat TAg cells along with an AP-1-responsive secreted alkaline phosphatase (SEAP) reporter plasmid [31]. 24 h after transfection the cells were stimulated with PMA and varying concentrations of $(CsA)_2$ or CsA-M for 24 h, after which SEAP activity was measured.

Figure 2



Synthesis of $(CsA)_2$ and CsA-M from CsA. NBS = N-bromosuccinimide, AIBN = 2,2'-azobis(2-methylpropiononitrile), $(Im)_2CO$ = carbonyldiimidazole.

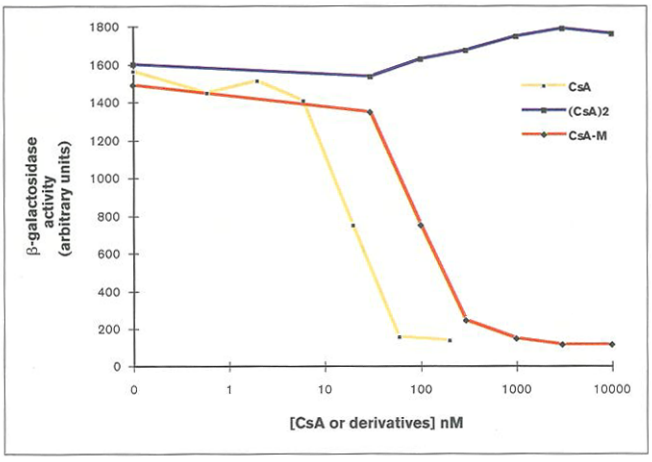
Figure 3



Fluorescence titration assays for $(CsA)_2$ and CsA-M with cyclophilin A. The fluorescence change observed for cyclophilin is plotted against the concentration of ligand added.

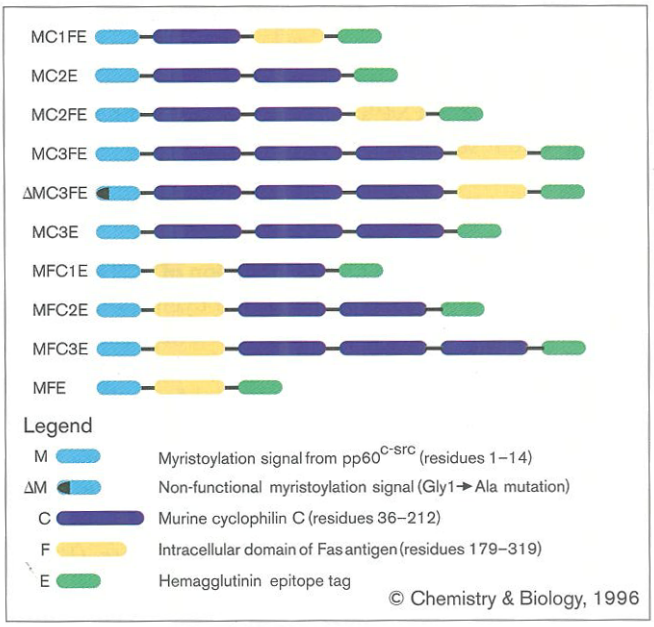
This provides an indirect assay for Fas signaling, since a reduction in the number of living cells decreases reporter gene expression. From the normalized data in Figure 6a it can be seen that all constructs in which the CyP domains precede Fas are capable of signaling, with MC3FE being the most effective. From the raw data in Figure 6b it can be seen that some of the constructs significantly reduce

Figure 4



Inhibition of NFAT signaling pathway by CsA, CsA-M and $(CsA)_2$ in Jurkat cells stably transfected with a β-galactosidase reporter gene containing three tandem NFAT response elements within the minimal IL-2 promoter [29]. In 96-well plates, 10^5 cells per well were stimulated with ionomycin (1 mM) and PMA (50 ng ml^{-1}) in the presence of varying concentrations of ligand. After 24 h, cells were assayed for β-galactosidase activity.

Figure 5



Schematic representation of DNA constructs encoding the Cyp–Fas fusion proteins used in this study.

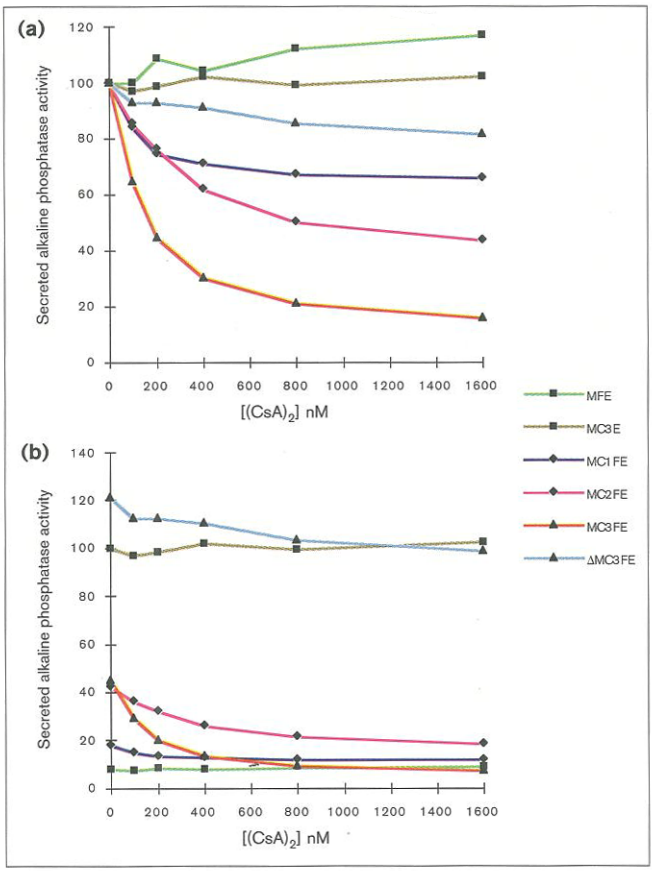
reporter gene expression even in the absence of $(CsA)_2$. Myristoylation was essential for activity, as ΔMC3FE was unable to signal. Western blots displaying the relative levels of expression of these constructs are shown in Figure 7. The constructs ΔMC3FE, MC2E and MC3E were expressed at high levels. MC3FE was expressed at a low level and the expression of MC2FE was barely detectable. MC1FE and MFE had undetectable levels of expression. To test whether dimerization of Fas was essential for MC3FE signaling in our assay, cells were treated with CsA-M and $(CsA)_2$ alone or in combination (Fig. 8). CsA-M had no effect on AP-1 signaling alone and $(CsA)_2$ in the presence of excess CsA-M was no longer able to induce signaling.

Based on the transient transfection data we decided to use MC3FE for the generation of stably transfected Jurkat T cell lines. Two such cell lines were derived and each showed >95 % apoptotic death following 24 h of stimulation with 100 nM $(CsA)_2$. The mode of death was clearly apoptotic, as assessed by an assay that reveals both cell morphology and viability (Fig. 9).

Discussion

In this study, we prepared conditional alleles (genes that produce a specific effect only under certain conditions) of Fas, encoding receptors designed to be activated by a CID derived from cyclosporin (Fig. 1). The early identification of Fas as a member of the TNFR superfamily suggested that receptor aggregation would initiate Fas signaling [32]. This expectation was supported by studies that used an

Figure 6



Triggering of cell death by $(CsA)_2$. The figure shows the results of transient expression experiments in Jurkat T cells stably transformed with large-T antigen. Cells were electroporated with a plasmid encoding a SEAP reporter gene under the control of three tandem AP1-binding sites (AP-1-SEAP, 1 μg) along with an excess (5 μg) of a plasmid encoding the immunophilin fusion construct. After 24 h, the cells were stimulated with PMA (50 ng ml^{-1}) and $(CsA)_2$. At 48 h the cells were assayed for SEAP activity. **(a)** Data for each construct normalized relative to $[(CsA)_2] = 0$. **(b)** Data for each curve normalized relative to $[(CsA)_2] = 0$ for the MC3E construct. Each data point represents the average of triplicate experiments.

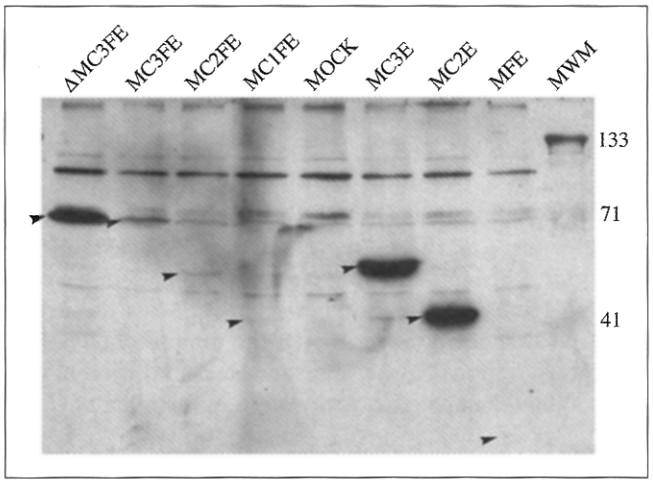
antibody to crosslink the receptor [33] and a parallel study in which Fas chimeras responsive to FK1012 were used [19]. Controlling the Fas pathway with a cell-permeable CID was expected to facilitate an understanding of the mechanism of signaling, and has provided a way to selectively ablate cells targeted to express the conditional allele, for example, by using cell-specific promoters in transgenic animals [19].

CsA is a natural CID, composed of immunophilin- and calcineurin-binding surfaces. To convert this natural heterodimerizer into a CID with two immunophilin-binding surfaces (a homodimerizer) and lacking a calcineurin-binding surface, we functionalized CsA at a position critical for calcineurin-binding and in a way that would enable two CsA molecules to be joined via a covalent linker. We chose a route that selectively derivatizes the terminus of the MeBmt-1 sidechain [26]. From previous studies, it was known that modifications at this site produced variants of CsA that no longer bind calcineurin, yet still retain the ability to bind CyP [34]. The synthesis outlined in Figure 2 provides $(CsA)_2$ in 6 steps and 30 % overall yield from CsA. As anticipated, a cellular signaling assay showed that even at concentrations as high as 10 μm, $(CsA)_2$ has no significant effect on the ability of PMA and ionomycin to stimulate the transcription factor NFAT, a process that requires the activation of calcineurin. Surprisingly, the monomeric version of the CsA dimer, CsA-M, showed some inhibition in this assay, although at a higher concentration than that of CsA.

To examine the properties of $(CsA)_2$ further, we tested whether this homodimerizer could bind to two CyP domains simultaneously. A fluorescence binding assay was used under conditions where the concentration of protein was greater than the expected K_d for CyP–CsA, resulting in a titration binding assay. The binding curves show clearly that two equivalents of CyP bind one equivalent of $(CsA)_2$ as the fluorescence approaches saturation when the concentration of $(CsA)_2$ is ~50 % of the concentration of CyP, whereas for CsA-M saturation occurs when the concentrations are equal.

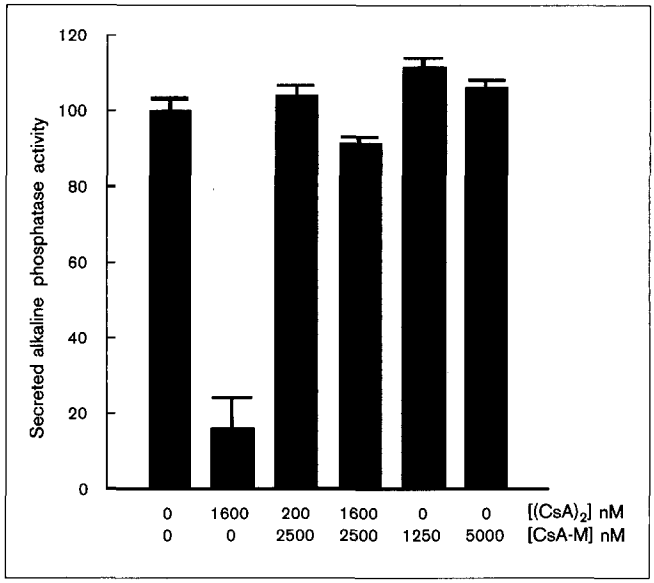
In a transient transfection assay for Fas signaling (Fig. 6a), we found that constructs containing one, two, or three CyP domains preceding the intracellular Fas domain (MCnFE, n=1,2,3) could activate the Fas signaling pathway in response to $(CsA)_2$; the MC3FE receptor was most effective. In contrast, the monomeric ligand, CsA-M, alone had no effect on the PMA-induced AP-1 reporter gene in cells transfected with MC3FE and was able to prevent $(CsA)_2$-induced signaling when added in excess (Fig. 8), indicating that dimerization is required for signaling. Constructs lacking the Fas domain or containing a non-functional myristoylation signal were unable to signal, demonstrating that membrane association is required for signaling. These results were fully consistent with similar experiments using FK1012. Constructs containing the Fas domain alone, or in which the Fas domain preceded the cyclophilin domains had no apparent activity. This was probably due to an auto-signaling effect since cells co-transfected with an excess of these constructs and a reporter gene showed little or no reporter gene activity when stimulated with ionomycin and PMA alone, suggesting that cells receiving both plasmids were eliminated by apoptosis during the 24-h recovery period (Fig. 6b). Western blots (Fig. 7) support this theory as the expression levels of Fas-containing constructs are inversely proportional to the level of ligand-independent signaling for each construct. Inactive and non-toxic constructs lacking the myristoylation signal or

Figure 7



Western blots of Jurkat-TAg cells transfected with plasmids encoding the indicated constructs. The anticipated positions of bands corresponding to the transfected plasmids are indicated with small arrows. 28 h after electroporation, soluble proteins in cell lysates were separated on a 10 % SDS-PAGE gel and transferred to nitrocellulose. Blots were probed with primary (anti-hemagglutinin antibody 12CA5, BABCO) and secondary (goat-anti-mouse horseradish peroxidase conjugate, Amersham) antibodies followed by ECL detection (Amersham).

Figure 8



Dimerization of the Fas construct MC3FE is essential for signaling. The figure shows the effect of $(CsA)_2$ and CsA-M on PMA-induced reporter gene activity in Jurkat-TAg cells transiently transfected with MC3FE and AP-1-SEAP, normalized to the value in the absence of ligand. Cells were assayed by the protocol detailed in the legend for Fig. 5. Experiments were done in triplicate; standard errors are indicated.

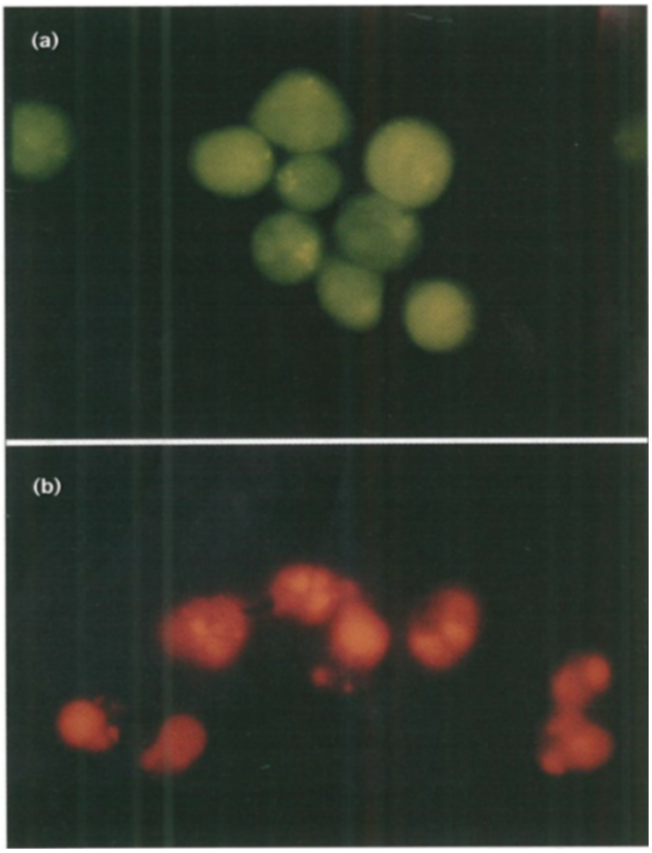
the Fas domain show a strong level of expression, further implicating Fas in cellular toxicity. Finally, the intracellular domain of Fas has been shown to self-associate and signal cell death [9,35].

Human Jurkat T-cell lines stably transfected with MC3FE clearly show the morphological features of apoptosis, with nuclear condensation and fragmentation visible within 6 h of treatment with $(CsA)_2$ (IC_{50} ~50 nM, Fig. 9). After 24 h of treatment with 100 nM $(CsA)_2$, ≥ 90 % cell death occurred as measured by Trypan Blue exclusion.

The crystal structure of the extracellular domain of TNFR complexed with its ligand shows that the complex is a trimer of the ligand bound to three receptor domains with C3 symmetry [36]. As there is extensive homology between the Fas ligand and TNF and between the extracellular ligand-binding domains of Fas and the TNFR, it has been hypothesized that Fas ligand induces Fas signaling through trimerization of Fas. Our results indicate that trimerization may not be necessary for signaling since the construct MC1FE is able to signal when treated with $(CsA)_2$, a process that can only directly result in the dimerization of Fas. However, we cannot rule out the possibility that dimerization results in a high affinity binding site for a third Fas intracellular domain. Viewed in light of the discovery of MACH1/FLICE, a pro-enzyme that must be proteolysed to release the active soluble ICE/CED-3-like protease domains, a simple model can be made in which dimerization of Fas mediates dimerization of MACH1/FLICE and activation of the protease. The proteolytic processing of MACH1/FLICE could occur by trans-proteolysis, analogous to the trans-phosphorylation induced by dimerization of receptor tyrosine kinases, where homodimerization increases the local concentration of these proteases, facilitating self-processing of MACH1/FLICE and delivery of an apoptotic signal.

The development of a second designed CID has opened up possibilities for the study of complex protein association events where multiple proteins are recruited to one or more sites in a cell. The CyP–CsA system and, in particular, the active carbamate of CsA (**2**, Fig. 2) has also been used in the synthesis of a heterodimeric CID, FKCsA, a molecule that can bind FKBP and CyP simultaneously [37]. FKCsA allows the specific heterodimerization of two disparate proteins to be accomplished with high specificity [37]. Because membrane association is required for Fas signaling and cellular toxicity, cell death can also be controlled through the inducible recruitment and aggregation of cytosolic Fas-containing proteins to a membrane docking protein with FKCsA [37]. This approach may provide a more tightly regulated death signal since the non-myristoylated Fas-containing proteins do not cause cell death through auto-association. FKCsA was also used to recruit a cytoplasmic protein to the nucleus and a transcriptional activation domain to a target gene [37]. We anticipate that, using the new and

Figure 9



$(CsA)_2$ induces apoptosis in Jurkat cells stably transfected with Cyp-Fas fusion proteins. Cells were photographed in growth media containing propidium iodide (50 μg ml^{-1}) and acridine orange (500 ng ml^{-1}). Acridine orange (green) accumulates in living cells with an intact membrane. Propidium iodide (red) stains DNA of dead or dying cells with leaky membranes. **(a)** Human Jurkat cells stably transfected with MC3FE plasmid. **(b)** Jurkat cells stably transfected with MC3FE plasmid treated with 500 nM $(CsA)_2$ for 24 h.

orthogonal CID-dimerization domain pair developed here together with the FKBP12–FK1012 system and the heterodimerizer FKCsA, we should now be able to independently activate multiple distinct pathways in a single cell or organism. For example, we now have tools to investigate signaling pathways for synergistic effects where multiple signaling proteins are independently activated or recruited to a common docking protein.

Significance

Cell-permeable CIDs can be used as equivalents of gain-of-function conditional alleles. The rationally designed CID synthesized in this study, $(CsA)_2$, has been used together with a rationally designed conditional allele of the Fas receptor. The synthesis begins with the natural CID, CsA, and illustrates the strategy of creating new CIDs by synthetically joining protein-binding surfaces. The conditional allele encodes a fusion protein composed of a short N-myristoylated region followed by one to three CyP dimerization domains and the intracellular tail of the Fas receptor. When stably expressed in a human tumor T-cell line, the artificial receptor can be dimerized and activated with the synthetic $(CsA)_2$ CID, resulting in apoptotic death of the T cells. Thus, a non-toxic molecule can be used to induce a lethal response in targeted cells. This cell ablation technique has already been shown to be useful in transgenic animals when combined with cell- or tissue-specific promoters, and it might even be useful in the treatment of tumors with non-toxic molecules when combined with gene delivery techniques (gene therapy).

Materials and methods

Fluorescence binding assay

Fluorescence measurements were made on a Hitachi F2000 fluorescence spectrometer following a 10 min incubation with ligand at 20 °C. Excitation was at 280 nm, 10 nm bandwidth and 33/100 filter. Emissions were scanned at 300–400 nm, 10 nm bandwidth. CyPA was diluted to 300 nM in 3 ml Tris buffered saline pH 7.4. Small aliquots of ligand in 20 % ethanol were added between successive measurements such that the total volume added was <1.5 %.

Cell lines and tissue culture

Jurkat-TAg [31] and Jurkat-NFAT-Z [29] cells were maintained in RPMI 1640 media containing 10 % (v/v) fetal calf serum, L-glutamine and penicillin/streptomycin. For transfections, 10^7 cells were electroporated (BTX 600 electroporator, 250 V, 800 μF, 129 Ω, 4 mm cuvettes in a total volume of 220 μl of RPMI 1640) with 10-min incubations at room temperature before and after electroporation. Following each electroporation, cells were diluted to 10 ml in media and incubated overnight at 37 °C, pelleted, washed, resuspended in 4 ml of medium and aliquoted into 96-well plates.

SEAP assays

Cells in 96-well plates were heated at 65–70 °C for 1.5 h to denature background phosphatases. 100 μl aliquots were transferred from each well to new plates containing 100 μl of a solution of 4-methyl-umbelliferyl phosphate (1 mM, Sigma) in 2M diethanolamine bicarbonate pH 10 and incubated at 37 °C for 2–10 h.

β-galactosidase assays

Cells in 96-well plates were pelleted by centrifugation. Following aspiration of the medium, pellets were dissolved in 150 μl of a solution of 4-methylumbelliferyl-β-D-galactoside (0.6 mM, Sigma) in 66 mM phosphate, 6.6 mM KCl, 0.66 mM $MgSO_4$, 0.1 % Triton X-100, pH 7. After incubating at 37 °C for 2–10 h, aliquots were quenched with 75 μl of 300 mM glycine, 15 mM EDTA pH 11.2. Fluorescence measurements were made using a Fluoroscan II fluorescence plate reader (ICN).

MeBmt(OH)-η-OCO-Im¹-CsA

MeBmt(OH)-η-OH^1-CsA (38 mg, 31 μmol, 1218.6 g mol^{-1}) and carbonyldiimidazole (20 mg, 4 eq., 124 μmol, 162.15 g mol^{-1}) were transferred into a 10-ml round bottom flask and dissolved in dry tetrahydrofuran (2 ml). Diisopropylethylamine (22 μl, 4 eq., 125 μmol, 129.25 g mol^{-1}) was added and the solvent was removed on a rotary evaporator at room temperature. The residue was purified by flash chromatography on silica gel using 0–20 % acetone in ethyl acetate as eluent to give 32 mg (78 % yield) of a white solid.

$(CsA)_2$: xylylenediamine CsA dimer

MeBmt(OH)-η-OCO-Im^1-CsA (12.5 mg, 9.52 μmol, 1312.7 g mol^{-1}) was dissolved in dichloromethane (200 μl). To this solution was added

22 µl (0.5 eq., 4.75 µmol) of a solution of xylylenediamine (14.7 mg, 136.2 g mol^{-1}) in dimethylsulfoxide (0.5 ml) and the reaction mixture was stirred for 72 h at room temperature under a nitrogen atmosphere. The reaction was diluted with acetonitrile (2 ml) filtered through glass wool and purified by reverse-phase high-pressure liquid chromatography (Beckman C18, 10 µ, 100 Å, 1 cm x 25 cm, 5 ml min^{-1}, 50 to 90 % acetonitrile/water (+0.1 % trifluoroacetate) over 30 min, 70 °C) to give 6.1 mg (49 % yield) of $(CsA)_2$ as a white solid.

Acknowledgements

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