Mechanistic studies of a signaling pathway activated by the organic dimerizer FK1012

Martin N Pruschy[1], David M Spencer[2], Tarun M Kapoor[1], Hiroshi Miyake[1], Gerald R Crabtree[2], and Stuart L Schreiber[1]*

[1]Howard Hughes Medical Institute, Department of Chemistry, Harvard University, Cambridge, MA 02138, USA and [2]Howard Hughes Medical Institute, Departments of Developmental Biology and Pathology, Stanford University Medical School, Stanford, CA 94305, USA

Background: The T-cell receptor (TCR) signaling pathway is initiated by regulated association of TCR chains, including the ζ chain. A recently reported method for inducing the dimerization or oligomerization of targeted proteins in cells used the TCR pathway as a test system. In cells transfected with cDNA encoding MZF3E, a chimeric receptor comprising the intracellular domain of the ζ chain and three copies of FK506-binding protein (FKBP), low concentrations of a synthetic dimer of the natural product FK506 (FK1012) activated the expression of reporter genes. We set out to examine the signaling pathway initiated by FK1012.

Results: We characterized the effect of FK1012 on MZF3E and a second chimeric receptor, MZF1E, which contains the ζ chain and one copy of FKBP. Only MZF3E gave FK1012-activated signaling, as shown by an increase in the kinase activity associating with MZF3E, and the appearance of specific phosphotyrosine-containing proteins. Signaling required localization of MZF3E to the inner plasma membrane, and activation of gene transcription in response to FK1012 was dependent on the protein phosphatase calcineurin and the transcriptional activator NF-AT. Some signaling events in the pathway had different kinetics when activated by MZF3E instead of the TCR, however. An unexpected requirement for the prolonged activation of calcineurin was observed.

Conclusions: Synthetic dimerizers can be used to gain control over cellular processes that require the association of specific intracellular proteins. The TCR signaling pathway was selected as an initial test system; we show here that one can indeed activate this signaling pathway by inducing the oligomerization of the cytoplasmic tail of the ζ chain with the cell-permeable reagent FK1012.

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Introduction

Regulated protein dimerization or oligomerization is important in many cellular processes. In many instances dimerization does not have to be geometrically precise [1], but instead simply creates a high local concentration of a particular protein at a particular site, thereby enhancing its activity. One example of this principle is illustrated by the current view of how transcriptional activators work. By binding simultaneously to a general transcription factor (for example, a TATA-box binding protein (TBP)-associated factor) and to its enhancer sequence, an activator can facilitate the complete formation of an initiation complex at a neighboring promoter sequence [2,3]. Examples of regulated protein recruitment are also seen in intracellular signaling pathways. The Ras pathway, for example, involves several contingent protein heterodimerizations (such as formation of the Ras•GTP–Raf complex) that result in the translocation of signaling proteins to sites that increase the probability of encounters with their substrates [4,5].

We have recently proposed a general method for inducing intracellular protein dimerization using low molecular weight organic ligands as dimerizing agents. The method was first illustrated with a test system involving the T-cell receptor (TCR) signaling pathway [6]. We now report a detailed investigation of this system that illustrates the ability of dimerizing agents to intercede in an endogenous pathway and to subvert it for the purpose of activating the transcription of a target gene.

The TCR signaling pathway

The TCR is a complex receptor. It contains two variable ligand-binding chains (α and β; see Fig 1), which are non-covalently associated with the invariant CD3 and ζ-subunits to form an eight-chain complex ($\alpha\beta\gamma\delta\varepsilon_2\zeta_2$). Regulated protein association is central to the TCR signaling pathway (for reviews see [7,8]). When TCRs are induced to aggregate by an antigen-presenting cell, the cytoplasmic tails of the associated chains (CD3 and ζ, of which ζ is probably the most important), are brought into close proximity. This leads to the phosphorylation of TCR subunits by protein tyrosine kinases (Fyn or Lck) of the Src kinase family, which are constitutively bound either to TCR subunits or to the CD4 and CD8 co-receptors [9,10]. A similar signaling pathway can be initiated by crosslinking the TCR complex using anti-CD3 antibodies.

Although the molecular basis for the increased phosphorylation of TCR subunits is still not clear, the aggregation may create a new microenvironment for a preassociated

*Corresponding author.

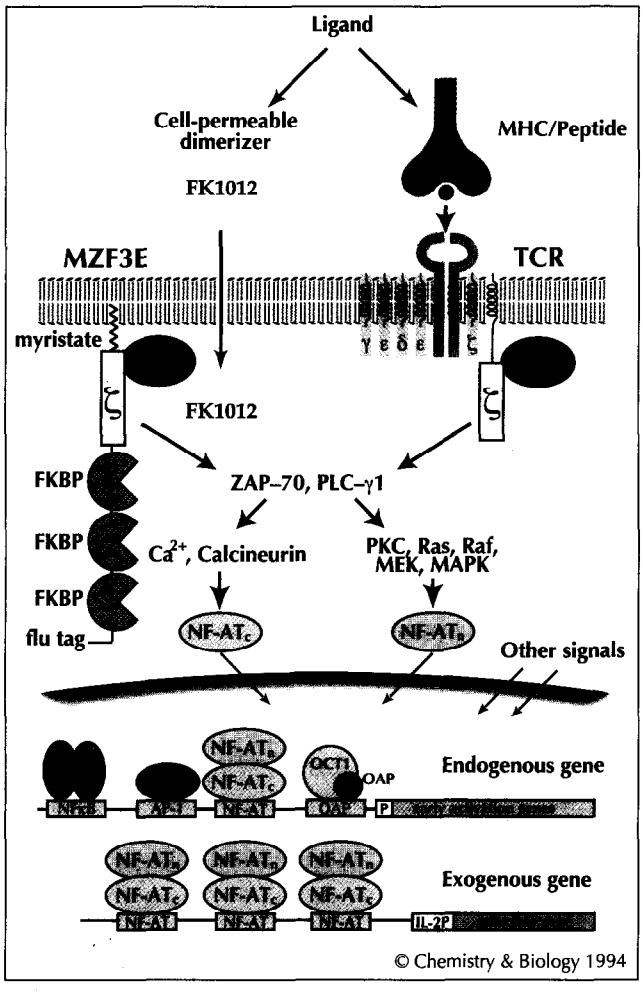

Fig. 1. Model for the intracellular signaling pathway activated by the TCR and the MZF3E receptor. Abbreviations are: MHC, major histocompatibility complex; ZAP-70, ζ-chain associated 70-kDa protein; PLC-γ1, phospholipase C-γ1; PKC, protein kinase C; MAPK, MAP kinase; MEK, MAPK/ERK kinase; NF-AT_c,NF-AT_n, cytoplasmic and nuclear components of NF-AT; OCT1, octamer factor 1; OAP, octamer associated protein, AP-1, activation protein 1; P, promoter; IL-2P, minimal IL-2 promoter.

kinase, leading to its activation via a conformational change or a *trans*-phosphorylation event. Alternatively, the pre-associated kinases may phosphorylate ζ continuously; aggregation would then serve simply to concentrate these kinases. In this model, phosphates would normally be removed from ζ by cellular phosphatases which would not be included in the TCR aggregate.

Tyrosine phosphorylation of ζ results in the creation of high-affinity docking sites for the cytosolic tyrosine kinase ZAP-70 [11]. ZAP-70 contains two SH2 domains that bind to phospho-Tyr-X-X-Leu sequences [12], and is thus recruited to the inner leaflet of the plasma membrane by heterodimerization with phospho-ζ. The membrane-localized ZAP-70 is thought to participate in the activation of phospholipase C (PLC)-γ. Tyrosine phosphorylation of PLC-γ activates its ability to cleave its membrane substrate, phosphatidyl-inositol-4,5-biphosphate, into the 'second messengers' diacyl glycerol (DAG) and inositol triphosphate (IP_3). DAG activates a protein kinase C (PKC) pathway, contributing to the activation of the 'MAP kinase pathway', possibly by phosphorylation of the serine-threonine kinase Raf, while IP_3 leads to the release of calcium and the subsequent activation of the calcium-activated protein phosphatase calcineurin [13–17].

After calcineurin is activated, dephosphorylation of NF-AT_c, the cytoplasmic subunit of the transcriptional activator NF-AT, causes it to translocate into the nucleus, where it encounters its partner NF-AT_n. NF-AT_n appears to be a member of the basic leucine zipper family of dimeric transcription factors (which also includes c-Jun and c-Fos) and is itself activated by the MAP kinase pathway [18,19]. Overall, therefore, there is a bifurcation in the TCR pathway after PLC-γ; the pathway then converges in the nucleus in the formation of the NF-AT_c/NF-AT_n complex (see Fig. 1).

This complex, the NF-AT transcriptional activator, cooperates with other activators, including those deriving from other signaling pathways, to activate transcription of early activation genes including interleukin-2 (IL-2), IL-3, IL-4, granulocyte-macrophage colony-stimulating factor and CD40L. IL-2 can heterotrimerize components of its receptor, in turn activating a second membrane–to–nucleus pathway ([20]; for review see [21]). This second signaling cascade is required for the T cell to progress through the first phase of the cell cycle, leading eventually to cell division.

Gaining control over the signaling pathway

Chimeric proteins containing the intracellular domain of the ζ-chain induce signaling when aggregated using antibodies, showing that aggregation of ζ is sufficient to initiate the TCR signaling pathway [22,23]. We have extended these results using the recombinant receptor MZF3E, which consists of several modular elements. The amino-terminus of MZF3E contains a 14-amino-acid sequence that serves as a substrate for myristoyl transferase [24]. Myristoylation of MZF3E was expected to facilitate its association with the plasma membrane [25]. After the myristoylation site (M) MZF3E contains the intracellular domain of the TCR ζ chain (Z) linked to three tandem copies of the immunophilin FKBP12 (F). The immunophilin modules serve as receptor domains for ligand-induced dimerization. An influenza hemagglutinin epitope (E), recognized by the monoclonal antibody 12CA5, forms the carboxyl-terminus of the receptor (see Fig. 1). The cellular expression of the cDNA encoding this fusion construct is driven by the constitutive promoter SRα of the eukaryotic expression vector pBJ5 [26].

FKBP12 belongs to a class of immunophilin proteins, originally discovered because of their high affinity for immunosuppressive drugs. FKBP12 binds to the natural products FK506 and rapamycin with high affinity (K_ds = 0.4 nM and 0.2 nM, respectively) The protein has intrinsic peptidyl-prolyl *cis–trans* isomerase (rotamase) activity [27,28], which is blocked on binding to either

FK506 or rapamycin, but which does not appear to be related to the ability of these molecules to inhibit intracellular signaling pathways. Instead, their actions are mediated by the formation of composite surfaces [29] in the FKBP12–FK506 and FKBP12–rapamycin complexes that allow binding to calcineurin [30] and the lipid kinase, FKBP–rapamycin-associated protein (FRAP) [31,32], respectively.

Inhibiting the function of calcineurin and FRAP results in the inhibition of different signaling pathways. As calcineurin mediates signaling from the TCR, FK506 blocks TCR-mediated signaling, and therefore prevents the activation of T cells. Interestingly, the immunophilin cyclophilin A, which has a rotamase activity similar to that of FKBP, binds a different immunosuppressive drug, cyclosporin A (CsA) [33], creating a different composite surface that also inhibits calcineurin [30]. Studies of FK506 and CsA reveal that they have two protein-binding surfaces, an immunophilin-binding surface and a calcineurin-binding one; they can thus be termed 'chemical inducers of dimerization' (CIDs). Though structurally unrelated, FKBP12 and cyclophilin A gain the same function upon binding to their respective ligands, namely, the ability to bind calcineurin (Fig. 2).

Two factors that were important in the selection of FK506 as a building block for a designed CID were its ability to cross cell membranes and its high affinity for FKBPs. To construct an FK506 dimer, two FK506 monomers were dimerized via a functional group within the calcineurin-binding domain. Thus the dimer should still bind to FKBP12, but the complex of the dimer with FKBP12 should not bind to calcineurin and thus should not block TCR signaling [6]. The dimeric molecule, which we called FK1012, displayed a 1:2 binding stoichiometry to FKBP12 (K_ds = 0.1 nM and 0.8 nM) and did not inhibit the Ca^{2+}-dependent signaling pathway of activated T cells even at high concentrations. (FK1012 was previously named FK1012A [6].) An optimized synthesis of FK1012 is described in this report (Fig. 3; see Materials and methods).

The recombinant receptor MZF3E should therefore be aggregated by FK1012, and should then induce the TCR signaling cascade that is initiated by anti-CD3 antibodies. To test this hypothesis, we used a reporter gene assay that provides a measure of signaling induced by FK1012. A responsive promoter element was constructed to transcribe a target gene under the control of NF-AT alone. The reporter construct consists of three consecutive NF-AT-binding sites and a minimal IL-2 promoter (lacking the binding sites for other transcriptional activators found in either the endogenous IL-2 promoter or its upstream enhancer) linked to the coding sequence of the reporter protein lacZ (β-galactosidase; 'exogenous gene' in Fig. 1) [34]. Cells stably expressing this construct are named NFATZ.

MZF3E-directed gene expression has been examined in the human T-lymphocyte cell line Jurkat stably (this study) or transiently [6] transfected with the MZF3E and NF-AT-inducible reporter gene constructs. Sub-micromolar concentrations of FK1012 triggered the expression of the reporter gene to approximately the same extent as that observed when the TCR was activated with monoclonal antibodies against the TCR complex (this study) or when the TCR was bypassed with pharmacological agents [6]. The ζ-chain and the three FKBP12-modules within MZF3E were shown to be essential for optimal signal transduction [6]; a similar receptor that contains only one FKBP12 module (MZF1E), and mutated versions lacking a functional myristoylation sequence, did not show efficient signaling.

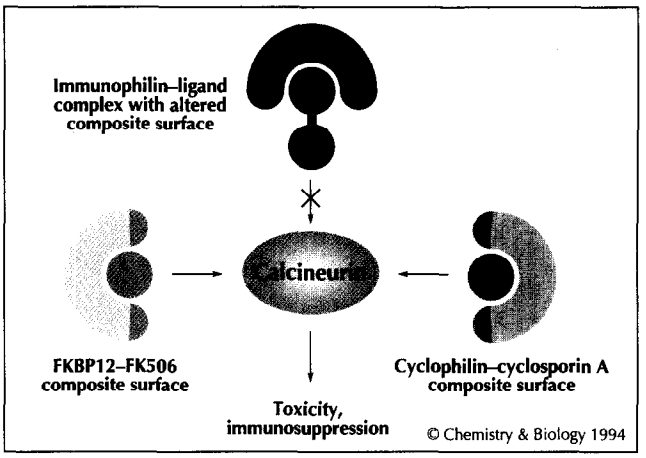

Fig. 2. Schematic illustration of the interaction of calcineurin with composite surfaces created by immunophilin–ligand complexes.

This study examines the ability of FK1012 to induce receptor aggregation and dissects the intermediate steps of the transmitted signal. We have shown that the designed receptor is coupled to the signal-transduction machinery used by its natural counterpart, the TCR, although some differences in the kinetics of the events that constitute the signaling cascade are seen. Transfecting the cDNA for the designed receptor MZF3E into T cells thus allows us to intervene in the endogenous pathway; furthermore, co-transfecting MZF3E with an inducible gene under the control of the transcriptional activator NF-AT allows us to subvert the signal in the nucleus and to activate an exogenous, targeted gene at will. The purpose of the present investigation was to determine the molecular basis for FK1012-dependent signaling.

Results and discussion

Localization of the recombinant receptor MZF3E

Signaling through the TCR requires its localization at the plasma membrane, since it is only here that the extracellular domains of the variable α and β chains of the TCR can interact with either MHC class I- or class II-peptide complexes on the surface of antigen-presenting cells. The cytoplasmic tails of chains associated with the TCR, γ, δ, ε and especially ζ relay the signal first to membrane-associated and then to cytosolic tyrosine kinases of the TCR

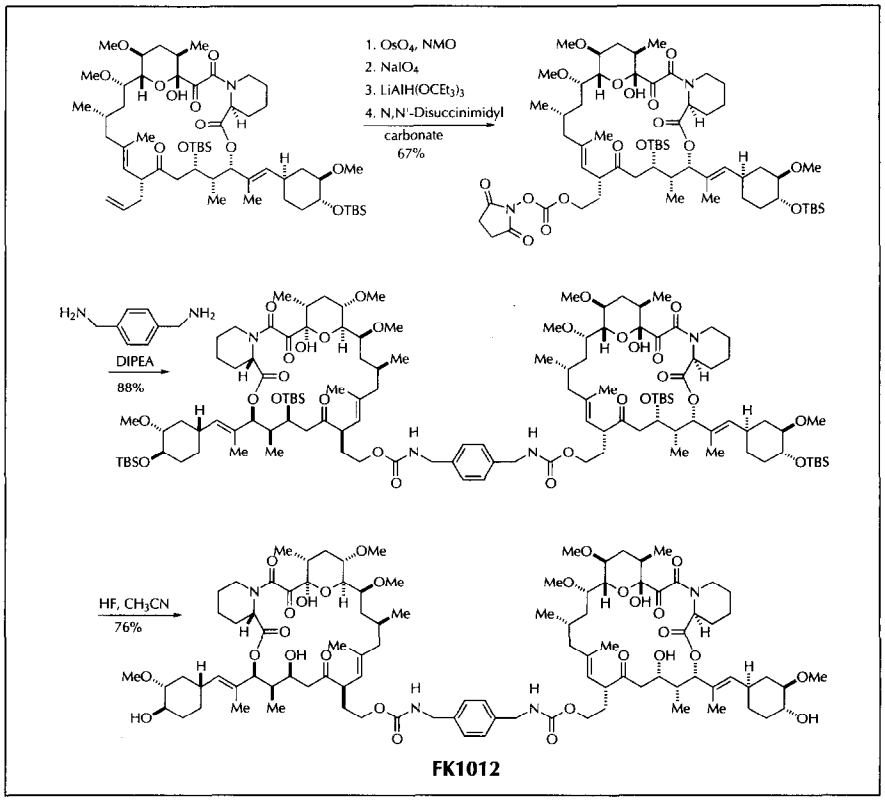

Fig. 3. Optimized synthesis of the dimerizing agent FK1012. Details of the synthesis are described in Materials and methods.

pathway. We compared the cellular localization of the expressed receptor MZF3E, which contains an intact Src-derived myristoylation sequence, with that of the mutant construct MΔZF3E in which the amino-terminal glycine was changed to alanine. This single amino-acid change in MΔZF3E has been shown to abolish receptor signaling [6] and the corresponding change in Src prevents its myristoylation [35]. MZF3E and MΔZF3E were expressed separately in Tag-Jurkat cells and their cellular localizations were determined using immunocytochemistry with a monoclonal antibody (mAb) against the carboxy-terminal influenza hemagglutinin (HA)-derived epitope tag. The MZF3E receptor was found to be associated with the plasma membrane, whereas the mutated MΔZF3E construct showed diffuse cytoplasmic staining indicative of cytosolic localization, outside the large nucleus of Jurkat cells. Only a minor fraction of the MZF3E receptor was associated with unidentified, presumably membranous intracellular structures (Fig. 4). These results confirmed that the amino-terminal glycine is essential for correct post-translational modification and, in conjunction with earlier results, that association with the plasma membrane is necessary for MZF3E-mediated downstream signaling.

FK1012-induced receptor aggregation

To demonstrate that intracellular protein association can be induced by a cell-permeable ligand, we developed a co-immunoprecipitation assay. The C-terminal influenza HA epitope tag (E_h, YPYDVPDYA) was exchanged with the Flag M2 epitope tag (E_f, DYKDDDDY) to generate two closely-related constructs, MZF3E$_h$ and MZF3E$_f$, which were coexpressed in Tag-Jurkat cells. The FK1012-induced heterodimerization of the constructs was confirmed by our ability to co-immunoprecipitate MZF3E$_h$ with MZF3E$_f$, using a mAb against the Flag M2 epitope tag (E_f). Western blots of the anti-Flag

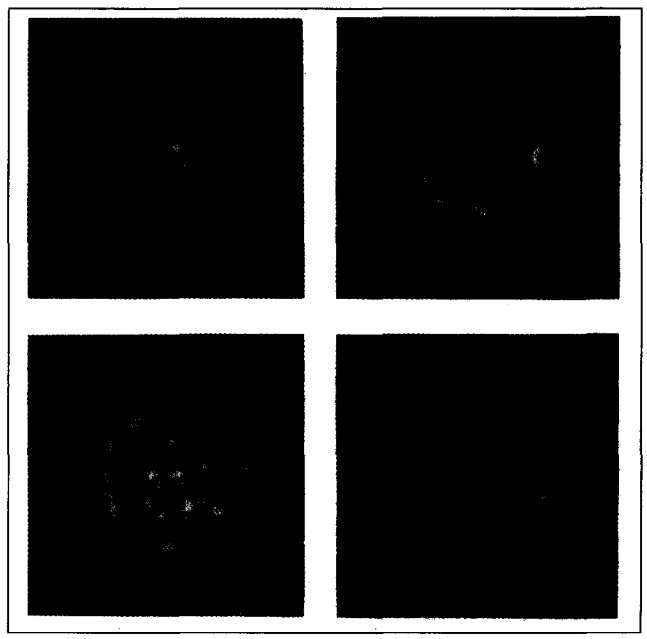

Fig. 4. Intracellular localisation of myristoylated MZF3E$_h$ and non-myristoylated MΔZF3E$_h$. Transiently-transfected Jurkat cells were stained with anti-HA mAbs, which react with the Eh epitope, as the primary antibody and fluorescein-labeled rabbit anti-mouse IgG as secondary antibody; top: phase contrast and fluorescence microscopy of MZF3E$_h$-expressing cell; bottom: phase contrast and fluorescence microscopy of MΔZF3E$_h$-expressing cell.

immunoprecipitates with anti-HA antibodies showed that the two constructs associate only when the cells have been pretreated with FK1012 (1 μM). In the absence of FK1012, no co-immunoprecipitation was observed (Fig. 5a). Although we cannot exclude the unlikely possibility that signaling occurs following intramolecular crosslinking of MZF3E [36], the results show that FK1012 does induce its intermolecular association.

We had demonstrated earlier that multiple FKBP12 domains on the myristoylated ζ chain were required for activation [6]. A recombinant receptor with only one FKBP12 domain, MZF1E, is unable to activate the intracellular signaling pathway. Nevertheless, co-immunoprecipitation experiments with $MZF1E_h$ and $MZF1E_f$ revealed that FK1012 induces the heterodimerization of these constructs as well (Fig. 5b). These experiments indicate that to initiate signaling, ζ needs to be aggregated, not merely dimerized, and that the failure of MZF1E to signal is not due to an inability to be dimerized by FK1012. In contrast to the ζ fusions, receptors that contain the cytoplasmic domain of a receptor that mediates an apoptotic pathway and a single immunophilin domain have been shown to signal upon simple dimerization (D.M.S. and G.R.C., unpublished data; P.J. Belshaw and S.L.S., unpublished data)

Interaction of protein tyrosine kinases with the MZF3E receptor

The intracellular subunits of the TCR chains have no intrinsic kinase activity. Nevertheless, aggregation by antigen-presenting cells or mAbs induces rapid tyrosine phosphorylation of intracellular proteins. The cytoplasmic tails of the CD3 subunits have been found to serve as docking elements for the intracellular tyrosine kinases Fyn and ZAP-70. While Fyn is physically associated with non-ligated and largely non-phosphorylated subunits of the TCR complex, ZAP-70 is recruited to the TCR complex and subsequently activated only after aggregation and tyrosine phosphorylation of these subunits [37].

To examine the interaction of MZF3E with protein kinases, we performed an *in vitro* kinase assay. Jurkat cells were transiently transfected with MZF3E and the expressed receptor was immunoprecipitated from cell lysates of untreated or FK1012-treated cells with the anti-HA mAb. The immunoprecipitates were incubated with low concentrations of $\gamma[^{32}P]$-ATP in the presence of $MnCl_2$ and analyzed on autoradiographs of SDS polyacrylamide gels to determine whether protein kinases associate with the receptor. A band corresponding to the molecular weight of 55 kDa was detected under all conditions tested. This substrate most probably represents the phosphorylated MZF3E receptor itself or an autophosphorylated, co-immunoprecipitated kinase (Fig. 6). An additional phosphorylated substrate that migrated with the molecular weight of ~70 kDa could be detected in immunoprecipitates of cells that were treated for 2 min with FK1012. Prolonged incubation (10 min) of the cells with FK1012 before cell lysis reduced the amount of ^{32}P incorporation in both bands. This diminished incorporation of radioactive phosphate might be due to the intracellular tyrosine phosphorylation occuring with non-radioactive (endogenous) ATP after prolonged treatment with FK1012, before cell lysis and immunoprecipitation. This would explain why we see reduced ^{32}P incorporation at a time (10 min) when tyrosine phosphorylation is maximal as determined by Western blotting of total cell lysates (Fig. 7). Treatment of the cells with a monomeric derivative of FK506 (FK506-M [6]), which binds to FKBP12 but does not form a complex capable of interacting with calcineurin, did not induce the phosphorylation of the additional substrate. Control experiments with the recombinant receptor MZE that lacks an FKBP12 domain failed to show any enhanced kinase activity.

To study the initiation of the MZF3E signaling pathway, we compared tyrosine phosphorylation activity of cells that had been activated through their TCRs or MZF3E receptors. Whole-cell lysates of cells stably transfected with both MZF3E and the NFATZ reporter gene construct (see Materials and methods) were analyzed at varying times after stimulation with either anti-CD3 mAb or FK1012 (Fig. 7). Western blots of the lysates were probed with the anti-phosphotyrosine mAb 4G10. A highly similar set of tyrosine-phosphorylated proteins was

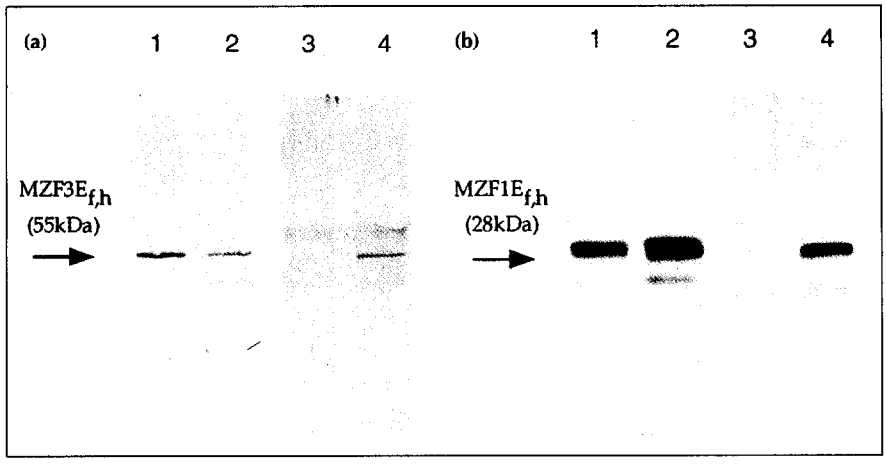

Fig. 5. Co-immunoprecipitation of ζ-chain constructs in the presence of FK1012. **(a)** $MZF3E_f$ and $MZF3E_h$ were coexpressed in Tag-Jurkat cells. Immunoprecipitations with Flag M2-mAb were performed after a 10 min incubation-period with (lanes 2, 4) or without (lanes 1, 3) FK1012. Immunoprecipitates were analyzed on western blots with anti-Flag M2 mAb (lanes 1, 2) or anti-HA mAb (lanes 3,4). **(b)** $MZF1E_{f,h}$-constructs were tested analogously to $MZF3E_{f,h}$ constructs. Immunoprecipitates were analyzed with anti-Flag M2 mAb (lanes 1, 2) or anti-HA mAb (lanes 3, 4) after a 10 min incubation period with (lanes 2, 4) or without (lanes 1, 3) FK1012.

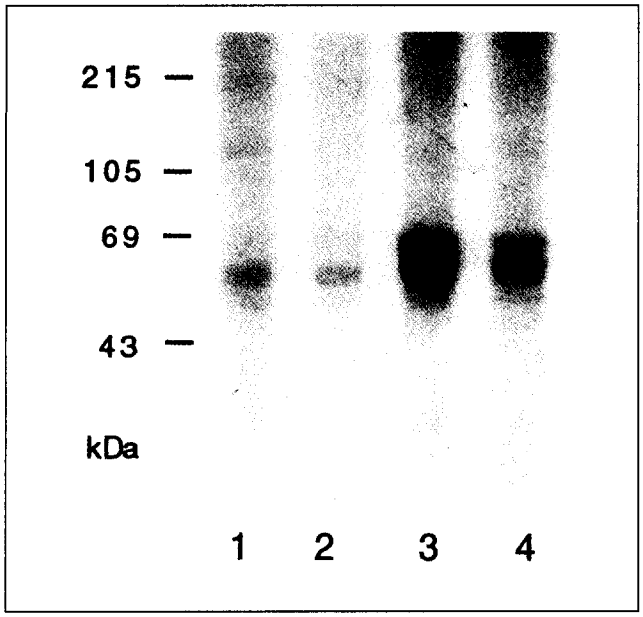

Fig. 6. Kinase activity associated with HA-immunoprecipitates from MZF3E transiently-transfected cells. MZF3E was immunoprecipitated from untreated cells (1), FK506-M (1 μM, 2 min) treated cells (2) or cells treated for 2 min (3) or 10 min (4) with FK1012 (1 μM). Immunoprecipitates were incubated with γ[^{32}P]-ATP and analyzed by SDS-PAGE.

observed in both cases, with a major band of 70 kDa and minor bands of 120 kDa, 62 kDa, 55 kDa and 42 kDa. FK1012 had no effect on Jurkat cells not expressing the MZF3E construct.

We suspect that MZF3E is physically associated with a kinase or kinases that coprecipitate with the non-ligated receptor and that receptor aggregation by FK1012 induces the recruitment and phosphorylation of additional proteins. The major tyrosine-phosphorylated substrate observed in the *in vitro* kinase assay is probably the cytosolic tyrosine kinase ZAP-70. Further experiments were performed to detect an interaction between MZF3E and ZAP-70. MZF3E was immunoprecipitated with anti-HA mAb from untreated and FK1012-treated cells and western blots of the immunoprecipitates were probed with anti-ZAP-70 polyclonal antibodies. ZAP-70 could only be detected in immunoprecipitates of FK1012-treated cells (data not shown), suggesting that the 70-kDa band in Fig. 7 is due to ZAP-70.

Although the set of phosphorylated proteins detected upon stimulation of the TCR was similar to that detected on stimulation of the MZF3E receptor, a significant difference was observed in the time course of the phosphorylation reactions. Tyrosine phosphorylation reached a maximum in 1 min after stimulation with anti-CD3 mAb. With FK1012, however, the maximum was detected only after 10 min. Delays have been reported in the signaling events induced by various other chimeric TCR-related constructs, but these delays were significantly shorter [38]. A possible explanation for the delayed phosphorylation is that the second binding event (leading to dimerization) may be slower in the case of FK1012 relative to the anti-CD3 antibody. In *in vitro* binding studies using human recombinant FKBP12 and FK1012, we have observed that while the formation of the 1:1 complex is rapid, the formation of the 2:1 complex requires at least several seconds [39]. Although we have not observed any interaction between MZF3E and endogenous FKBP12 using western blot analyses, such interactions could also delay receptor aggregation.

Calcineurin is a key element in the MZF3E pathway

The Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin [40] is central in the TCR signaling pathway [41]. This became clear when it was recognised that calcineurin is the target for both of the immunophilin–ligand complexes FKBP12–FK506 and cyclophilin A–CsA [30]. Other studies have shown that an increase in the concentration of intracellular Ca^{2+} regulates the activity of the transcription factor NF-AT by inducing the nuclear translocation of its cytosolic

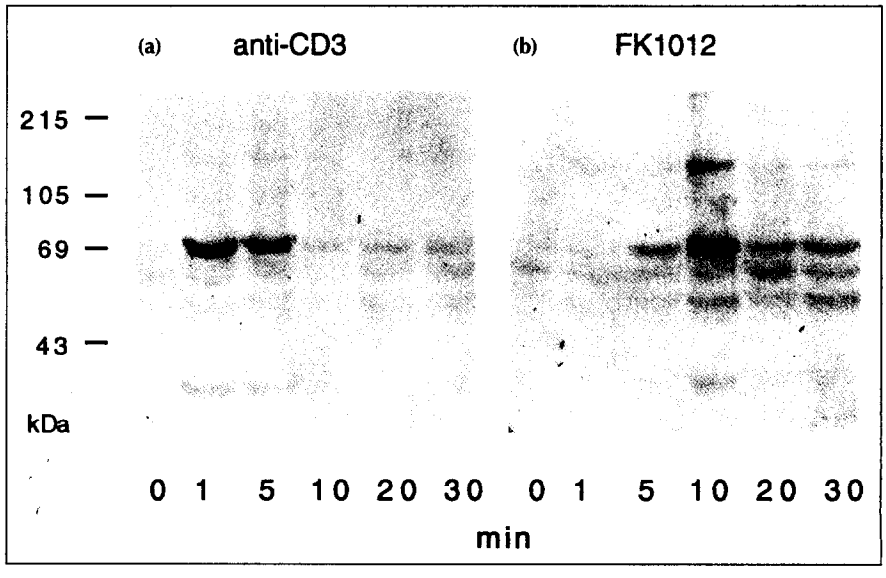

Fig. 7. Ligand-induced tyrosine phosphorylation. Stably-transfected MZF3E Jurkat cells were activated with FK1012 or an anti-CD3 mAb for various periods. Aliquots of whole cell lysates were analyzed on western blots with an anti-phosphotyrosine mAb.

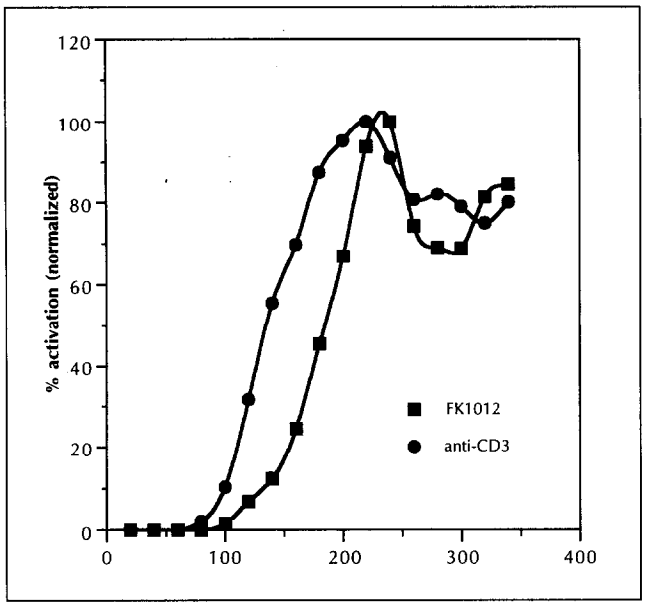

Fig. 8. Time course of β-Gal expression. Stably-transfected MZF3E NFATZ Jurkat cells were activated with FK1012 or an anti-CD3 mAb and aliquots were analyzed for β-Gal activity at different time points over 350 min.

subunit NF-AT_c [42]. This translocation appears to be regulated by calcineurin [41].

We studied the effect of calcineurin inhibition in MZF3E-NFATZ Jurkat cells that were stimulated with either anti-CD3 mAb or FK1012. The calcineurin inhibitor FK506 (5 nM), added at the time of receptor stimulation, completely inhibited the expression of the reporter protein β-galactosidase (β-Gal). To exclude a mechanism of FK506 inhibition that involves competition for binding to MZF3E (in other words, a de-dimerization effect), we determined that the FKBP12-independent calcineurin inhibitor CsA (25 nM) exhibited the same effect (data not shown). These results indicate that calcineurin is also required for FK1012-induced signaling.

Amplification of the delay along the pathway

We next determined whether the observed delay in tyrosine phosphorylation was manifested after the induced signal was transmitted from the plasma membrane to the nucleus. Stably-transfected MZF3E-NFATZ Jurkat cells were activated with anti-CD3 mAb or FK1012, and the time course of β-Gal expression was assayed by measuring the activity of the reporter gene. Half-maximal expression of β-Gal was observed 130 min after stimulation of the TCR with anti-CD3 mAbs. Half-maximal expression induced by FK1012 was seen 180 min after stimulation (Fig. 8). Thus, the delay observed in the tyrosine phosphorylation of proteins following receptor activation was not only preserved but actually amplified along later steps of the pathway.

As different clones exhibited different amounts of MZF3E expression, we normalized the data by comparing the amount of protein activity observed in response to FK1012 or anti-CD3 mAb, taking the maximum stimulation seen in response to stimulation by either agent as 100%. The level of β-Gal activity induced by FK1012 in different stably-transfected NFATZ Jurkat cell lines corresponded to ~40–60 % of the activity observed following TCR stimulation. Experiments performed using cells transiently co-transfected with MZF3E and the reporter gene construct usually resulted in greater levels of FK1012-induced activity relative to that observed with the anti-CD3 mAb.

To determine where in the signaling pathway the amplification of the delay occurs, we investigated the duration of calcineurin action required for signaling by studying the inhibition of calcineurin. If the signaling process is linear, it might be expected that after the calcineurin-dependent translocation of NF-AT_c was accomplished, CsA would no longer inhibit reporter gene expression. CsA (25 nM) was added after different intervals following stimulation of the TCR or MZF3E with anti-CD3 mAb or FK1012, respectively, and the resultant β-Gal activity was assayed 270 min after receptor stimulation. CsA inhibited the inducible appearance of β-Gal activity by up to 50 % even when added 75 min after the initial activation of the TCR. In the case of MZF3E activation, the period of CsA sensitivity was much longer, and greater than 50 % inhibition was observed 120 min after addition of the dimerizing agent (Fig. 9). These results demonstrate that events upstream of (or possibly involving) calcineurin are responsible for this delay in the FK1012-dependent signaling process.

Implications for TCR signaling

The time course of sensitivity towards CsA in the case of TCR stimulation is intriguing, since the intracellular Ca^{2+}-spike is observed as early as 1 min and dephosphorylated

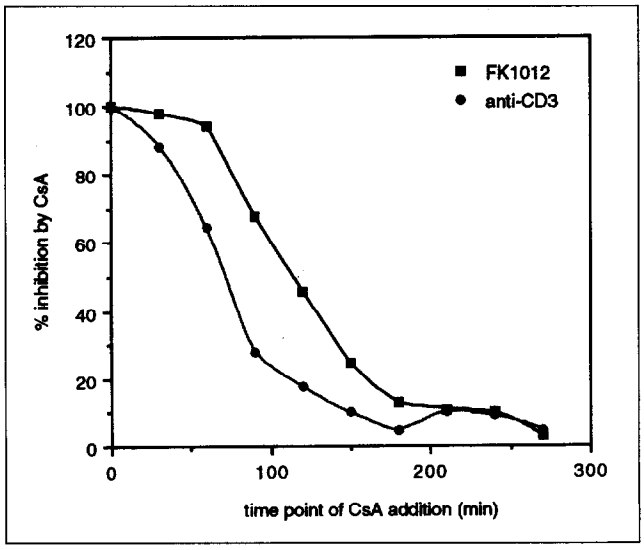

Fig. 9. CsA-dependent inhibition of β-Gal expression. Stably-transfected MZF3E NFATZ Jurkat cells were activated with FK1012 or an anti-CD3 mAb. CsA was added to different samples at the indicated time points and β-Gal activity of all samples was measured 4.5 h after initial cell stimulation. The graph shows the percentage of inhibition by CsA versus time of CsA addition.

NF-AT$_c$-subunits can be detected in the nucleus within 10 min after receptor stimulation. It is possible that calcineurin may be continuously required to promote the translocation of newly-synthesized NF-AT$_c$. Alternatively, the phosphatase might regulate other transcriptional activators involved in the endpoint of TCR signaling. For example, calcineurin appears to be involved in the activation of Jun kinase (Jnk), which may activate the Oct-1/Oct-1 associated protein (OAP) complex [43]. OAP has recently been shown to contain members of the Jun family of activators [44] and is therefore a substrate for Jnk. Like the NF-AT complex, Oct-1/OAP binds to the IL-2 enhancer and is required for transcription of the IL-2 gene [45]. Our results are also consistent with earlier studies of the mutant Jurkat T cell line JCAM1 [46]. These cells, which carry a defect in the tyrosine kinase Lck, fail to express IL-2 following TCR activation and are capable of producing only a transient burst of intracellular Ca^{2+}, in contrast to the persistent induction of Ca^{2+} in IL-2 expressing T cell lines. Thus, the failure of JCAM1 cells to produce a continuous supply of Ca^{2+} results in their inability to provide the prolonged activation of calcineurin required for maximal NF-AT-mediated and Oct-1/OAP-mediated transcription and thus IL-2 production.

Significance

Many important cellular processes require specific protein–protein interactions, and often these are under regulatory control [47]. We have developed a general method to induce such interactions in living cells with low molecular-weight organic compounds. These dimerizers (CIDs) have two covalently-linked immunophilin-binding surfaces. They are used to dimerize fusion proteins equipped with immunophilin domains.

In principle, inducible protein dimerization can be used to gain control over any process that is regulated by protein–protein association. The TCR signaling pathway is the first system in which this principle has been tested [6]. We have recreated the signaling activity of the TCR-associated ζ chain in the chimeric receptor MZF3E, which contains three copies of a domain that binds the natural product FK506. A designed dimer of FK506, FK1012, aggregates this receptor, initiating a contingent series of events that appears to be the same as that activated by the natural TCR. We have shown that both oligomerization and membrane localization are required for signaling.

In this system, only one receptor chain (the ζ domain in MZF3E) is involved in signaling; in the TCR, the γ, δ and ε chains of CD3 may also be involved. We therefore compared the detailed kinetics of signaling via MZF3E and via the TCR. We found that FK1012-dependent signaling is slower than signaling activated by anti-CD3 antibodies, yet results in a comparable level of gene activation. We also found that gene activation requires prolonged activation of calcineurin, which may reflect a requirement for calcineurin in the activation of at least two transcription complexes that are essential for transcription of the IL-2 and other early activation genes. Thus the ability to intervene in the signaling pathway of the TCR, as well as establishing the principle that intracellular protein association can be induced by a cell-permeable ligand, has provided insight into the nature of the signaling pathway itself.

Materials and methods

Cell culture and transfections

All cell lines were cultivated in RPMI 1640 medium supplemented with 10 % fetal bovine serum. Tag-Jurkat cells are a derivative of the Jurkat cell line stably transfected with the SV40 large T antigen [17]. The derivative cell line NFATZ contains a single stably-integrated construct that has three NF-AT-binding sites, a minimal IL-2 promoter and the lacZ sequence [34]. Stable MZF3E transfectants of NFATZ Jurkat cells were obtained by antibiotic selection using G418 (1 mg ml^{-1}) until resistant clones grew out. Transient transfections of Tag-Jurkat cells were performed by electroporation (BTX, 300 V, 800 μF) using 10 μg of each plasmid for 1 x 10^7 cells.

Construction of plasmids

Plasmids encoding the constructs MZF1E$_h$, MZF3E$_h$, MΔZF3E, MZF4E$_h$ have been described elsewhere [6]. Exchange of the influenza HA epitope-tag (E$_h$) in MZF1E$_h$ and MZF3E$_h$ was performed by replacing the sequence between the restriction sites, *Sal* I and *Eco* RI, by a small insert coding for the Flag-epitope-tag (E$_f$).

Immunoprecipitation and immunoblots

For whole cell lysate experiments, stably-transfected MZF3E cells (1 x 10^7 ml^{-1}) were activated for different time periods with 1 μM FK1012 or anti-CD3 mAb (1:500 dilution of ascites fluid; gift of J. Strominger, Harvard University). Activation was terminated by a short centrifugation step (30 s) and addition of suspension buffer (100 mM NaCl, 1 mM EDTA, 0.1 mM $NaVO_3$, 10 mM Tris-HCl, pH 7.6, 1 mM PMSF) followed by 2x sample buffer. Samples were boiled for 10 min and sonicated for 2 min in a sonicating bath. Aliquots corresponding to 2 x 10^6 cells were separated on polyacrylamide gels and transferred to nitrocellulose. Phosphotyrosine residues were detected using the mAb 4G10 (UBI, Lake Placid, N.Y.).

For crosslinking experiments, Tag-Jurkat cells were transiently cotransfected with MZF3E$_h$ and MZF3E$_f$. Twenty-four hours after transfection, the cells were incubated in the absence or presence of 1 μM FK1012 for 10 min at 37 °C, harvested by centrifugation and resuspended in 1 ml of lysis buffer (1 % NP-40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 3 μM pepstatin A, 2 μM leupeptin) for 30 min at 4 °C. Cell lysates were centrifuged at 10 000 g for 10 min at 4 °C and the supernatant was incubated with 30 μl anti-Flag M2 affinity gel (IBI, New Haven, CT). The affinity gel was washed twice with lysis buffer and the bound proteins were eluted from the matrix by incubation in 40 μl of Flag-peptide (1 mM) for 20 min at room temperature, separated on polyacrylamide gel electrophoresis and blotted onto nitrocellulose. Western blotting was performed using anti-Flag-M2 mAb (IBI) and anti-influenza HA mAb (12CA5, BABCO, Richmond, CA).

In vitro *kinase assay*

MZF3E transfected Tag-Jurkat cell (10^7 cells ml^{-1}) were lysed and immunoprecipitated with the anti-HA mAb and protein A agarose (Sigma Co., St Louis, MO) in the presence of 1 % NP-40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 3 μM pepstatin A, 2 μM leupeptin, 10 mM Na pyrophosphate, 0.4 mM EDTA, 0.4 mM Na orthovanadate, and 10 mM NaF. Immunoprecipitates were washed once with lysis buffer, twice with 2 M NaCl, 50 mM Tris-HCl, pH 7.4, twice with water, and resuspended in 25 μl of 20 mM Tris-HCl, pH 7.4, 10 mM $MnCl_2$, 10 μCi of γ [^{32}P]-ATP (>6 000 Ci $mmol^{-1}$) for 10 min at room temperature. The labeled complex was then washed twice with water and analyzed by SDS-gel electrophoresis and autoradiography [22].

β-galactosidase assay

Expression of the β-galactosidase reporter gene in the stably-transfected NFAT Jurkat cell line (14) was determined by assaying for β-galactosidase activity. NFAT cells stably transfected with MZF3E were stimulated with FK1012 or anti-CD3 MAb at 1 x 10^6 cells ml^{-1} in fresh media. Aliquots corresponding to 2 x 10^5 cells were centrifuged and the pellet was resuspended in 150 μl of reaction medium (100 mM Na_2HPO_4, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 0.1 % Triton X-100, 0.5 mM 4-methyl-umbelliferyl β-D-galactoside (Sigma). After 1 h incubation at 37 °C, the reaction was terminated by adding 75 μl stop buffer (300 mM glycine, 15 mM EDTA, pH 11.3) and fluorescence was measured with a fluorescence-spectrophotometer at 350 nm excitation and 450 nm emission wavelength [48]. The results are the mean values determined from three independent experiments.

Immunofluorescence cell staining

Transiently-transfected Tag-Jurkat cells were attached to poly-lysine coated coverslips, fixed in paraformaldehyde (4 %) and lysed with methanol. Cells were incubated with anti-HA mAb and washed with PBS. Following incubation with fluorescein-coated rabbit anti-mouse IgG secondary antibody, the cells were analyzed by fluorescence microscopy [49].

Synthesis of FK1012

1) Synthesis of the mixed carbonate of FK506

24,32-*Bis*(*tert*-butyldimethylsilyloxy)-FK506 (710 mg, 688 mmol) was dissolved in THF (20 ml) and water (1 ml) and charged with N-methyl-morpholine N-oxide (403 mg, 3.44 mmol, 5 equiv) and 0.157 M aqueous osmium tetroxide (876 ml, 138 mmol, 0.2 equiv). After stirring at room temperature for 3 h, the reaction was diluted with 50 % aqueous MeOH (6.7 ml) and sodium periodate (1.47 g, 6.87 mmol, 10 equiv). After 1 h the mixture was extracted with ether, washed with brine, dried and concentrated. The resulting crude aldehyde was dissolved in THF (20 ml) and treated with lithium tris[(3-ethyl-3-pentyl)oxy]aluminum hydride (2.07 ml, 1.04 mmol, 1.5 equiv) at −78 °C. After 40 min, the reaction was quenched with saturated NH_4Cl, dried with Na_2SO_4, and concentrated. The crude alcohol was then dissolved in CH_3CN (20 ml), 2,6-lutidine (800 ml, 6.87 mmol, 10 equiv) and N,N'-disuccinimidyl carbonate (881 mg, 3.44 mmol, 5 equiv). The mixture was stirred at room temperature for 19 h, diluted with saturated $NaHCO_3$, extracted with CH_2Cl_2, dried, concentrated, and subjected to flash chromatograpy (hexane/ethyl acetate; 3:1 to 2:1 to 1:1) to give the desired mixed carbonate (542 mg, 67 %).

2) Synthesis of protected FK1012

The mixed carbonate of 24,32-bis(*tert*-butyldimethylsilyloxy)-FK506 (200 mg, 170 mmol) was dissolved in CH_2Cl_2 (20 ml), and treated with diisopropylethylamine (59 ml, 339 mmol, 2 equiv) and *p*-xylylenediamine (17 ml, 85.0 mmol, 0.5 equiv, 5.0 mM solution in DMF) at room temperature. After 14 h, the reaction was treated with saturated $NaHCO_3$, extracted with CH_2Cl_2, dried and concentrated. The resulting oil was subjected to flash chromatography (hexane/ethylacetate; 3:1 to 2:1 to 1:1) to yield the *bis*(silyl ether) of FK1012 (169 mg, 88 %).

3) Synthesis of FK1012

24,32,24',32'-Tetrakis(*tert*-butyldimethylsilyloxy)-FK506 (163 mg, 72.1 mmol) was dissolved in CH_3CN (7.2 ml) and 49 % hydrofluoric acid (1.0 ml) at room temperature. After 8.5 h, the reaction was quenched by addition of saturated $NaHCO_3$, and extracted with CH_2Cl_2, dried and concentrated. The residue was purified by flash chromatography (CH_2Cl_2/methanol; 50:1 to 10:1, gradient elution) to give FK1012 (98.4 mg, 76 %). IR (film) 3424 m (OH), 2934 s, 2828 sh, 1713 s, 1649 s, 1522 w, 1453 m, 1096 s; ^{1}H NMR (400 MHz, C_6D_6) d 7.19 (mult), 5.76 (s), 5.57 (s), 5.48–5.32 (mult), 5.20 (d, J=9.0), 4.95 (d, J=3.6), 4.65 (d, J=10.9), 4.31–3.99 (mult), 3.78–3.45 (mult), 3.26 (s), 3.18 (s), 3.14 (s), 3.13 (s), 3.09 (s), 2.98–2.73 (mult), 2.70 (s), 2.38 (br s), 2.19–1.81 (mult) 1.68–1.01 (mult), 1.68 (s), 1.63 (s), 1.48 (s), 1.20 (d, J=6.5), 0.92 (d, J=7.0), 0.83 (s); ^{13}C NMR (100 MHz, C_6D_6) d 197.0, 169.1, 165.3, 157.0, 138.8, 138.6, 138.4, 138.3, 133.3, 122.1, 118.6, 108.0, 97.7, 84.5, 75.6, 73.9, 72.8, 70.5, 57.0, 56.3, 55.7, 49.4, 45.0, 40.5, 39.5, 35.2, 33.6, 33.0, 31.8, 31.0, 30.7, 28.4, 25.9, 24.8, 21.6, 20.4, 17.0, 16.8, 16.2, 14.3, 10.0; MS (FAB) Calculated for $C_{96}H_{146}N_4O_{28}Na$ (based on integer masses): 1825. Found: 1825 (30), 754.

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