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Controlling Signal Transduction with Synthetic Ligands

David M. Spencer, Thomas J. Wandless, Stuart L. Schreiber,* Gerald R. Crabtree*

Dimerization and oligomerization are general biological control mechanisms contributing to the activation of cell membrane receptors, transcription factors, vesicle fusion proteins, and other classes of intra- and extracellular proteins. Cell permeable, synthetic ligands were devised that can be used to control the intracellular oligomerization of specific proteins. To demonstrate their utility, these ligands were used to induce intracellular oligomerization of cell surface receptors that lacked their transmembrane and extracellular regions but contained intracellular signaling domains. Addition of these ligands to cells in culture resulted in signal transmission and specific target gene activation. Monomeric forms of the ligands blocked the pathway. This method of ligand-regulated activation and termination of signaling pathways has the potential to be applied wherever precise control of a signal transduction pathway is desired.

Biological specificity usually results from highly specific interactions among proteins. This principle is exemplified by signal transduction, the process by which extracellular molecules influence intracellular events. Many pathways originate with the binding of extracellular ligands to cell surface receptors. In many cases receptor dimerization leads to transphosphorylation and the recruitment of proteins that continue the signaling cascade. The realization that growth factor receptors could be activated by homodimerization resulted from the observation that receptors could be activated by antibodies that crosslinked two receptors (*1*). Subsequently, many receptors were found to have the same properties. The extracellular and transmembrane regions of many receptors apparently function by bringing the cytoplasmic domains of the receptors in close proximity by a ligand-dependent dimerization or oligomerization, whereas the cytoplasmic domains of the receptor convey specific signals to internal compartments of the cell.

We have devised a general method for investigating the role of protein homodimerization, heterodimerization, and oligomerization in vivo. Signaling domains of receptors were intracellularly expressed as fusion proteins with a specific dimerization domain. We focused on the immunophilins (*2*) as the dimerization component of the fusion protein. Treatment of the cells with a cell permeable reagent comprised of two immunophilin ligands linked by a covalent tether was used to initiate dimerization. We constructed an artificial, myristylated receptor that lacked extracellular and transmembrane domains and induced its aggregation with our synthetic dimerization ligand. In analogy to other chimeric receptors (*3*), the receptor was designed to propagate an intracellular signal through subsequent protein-protein intractions and thereby activate a specific subset of transcription factors, whose actions were detected with a reporter gene assay. We demonstrated that a transmembrane domain was not essential for signaling, although membrane attachment was required. Intracellular crosslinking of proteins by synthetic ligands has potential in basic investigations of a variety of cellular processes and in regulating the in vivo synthesis of proteins of therapeutic or agricultural importance.

Elimination of FK506 inhibitory activity toward calcineurin by dimerization. Ideal characteristics for a molecular matchmaker include lipid solubility, lack of untoward cellular actions leading to toxicity, high affinity binding to its target receptor, and metabolic stability (particularly if oral administration is desired). The FK506 molecule was considered as a precursor to such a molecule (Fig. 1). It readily crosses cell membranes and binds to the immunophilin FKBP12 with high affinity, having a dissociation constant (K_d) of 0.4 nM (*4*), creating a new composite FKBP12-FK506 surface (*5*, *6*). The immunophilins, which

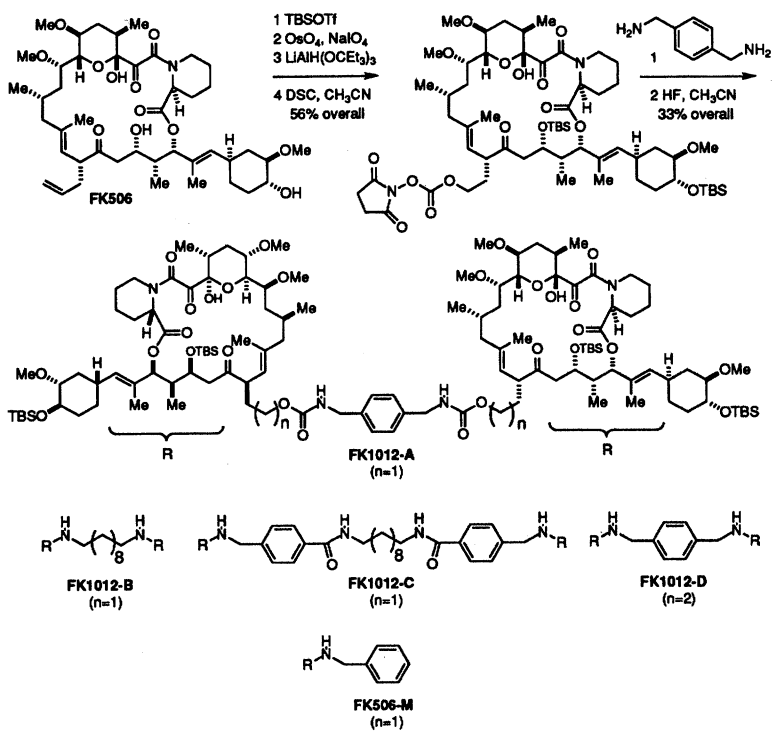

Fig. 1. Synthesis and molecular structures of FK1012 synthetic ligands. Reagents and conditions for the synthesis of FK1012s. Synthesis of mixed carbonate: (step 1) 4 equivalents (equiv) of TBSOTf, 5 equiv 2,6-lutidine, methylene chloride, 0°C, 1 hour; (step 2) 0.1 equiv OsO_4, 10 equiv $NaIO_4$, 5 equiv *N*-methylmorpholine-*N*-oxide, tetrahydrofuran, water, room temperature (RT) 5 hours; (step 3) 1.0 equiv lithium tris[(3-ethyl-3-pentyl)oxy]aluminum hydride, tetrahydrofuran, −78°C, 1 hour; (step 4) 5 equiv DSC, 10 equiv 2,6-lutidine, RT, 19 hours. Synthesis of FK1012-A: (step 1) 1.0 equiv mixed carbonate, 0.5 equiv para-xylylenediamine, 10 equiv triethylamine, acetonitrile, RT, 18 hours; (step 2) 10 percent (v/v) HF (as a 48 percent aqueous solution) in acetonitrile, RT, 16 hours. Abbreviations are TBS, tertiary-butyldimethylsilyl; OTf, triflate; Et, ethyl; DSC, *N,N'*-disuccinimidyl carbonate; Me, methyl.

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include the cyclophilins and FK506 binding proteins (FKBPs), are widespread and abundant proteins thought to participate in protein folding and intracellular transport similar to the chaperones. The FKBP12-FK506 complex binds to and inactivates calcineurin, a Ca^{2+}, calmodulin-dependent protein phosphatase (*7*). The inactivation of calcineurin results in impaired signaling of the T cell antigen receptor (TCR) and subsequent immunosuppression (*8–10*). In addition, inactivation of calcineurin underlies the toxicity of FK506 (*11*).

To convert FK506 into a nonimmunosuppressive and nontoxic matchmaker, we needed to impair its ability to interact with calcineurin by modifying the appropriate moieties, and also allow for the linking of two (or more) FK506 molecules. However, these modifications could not interfere with FKBP12 binding. Previous investigations have revealed that the allyl substituent of FK506 is an important element for calcineurin-binding (*12, 13*). Thus, several dimeric FK506 variants termed FK1012s were synthesized by the route shown in Fig. 1.

The FK1012s were examined for their ability to inhibit signal transmission in the T cell line Jurkat-TAg, which is sensitive to FK506 (*14, 15*) (Fig. 2). Inhibition of signal transduction was measured as a reduction in secreted alkaline phosphatase (SEAP) under the control of an NF-AT–responsive promoter element in the reporter plasmid NF-AT-SX (*8*). Nuclear factor of activated T cells (NF-AT) is an inducible, T cell–specific transcription factor that binds to the enhancers of a number of lymphokine genes, including interleukin-2 (IL-2), and is necessary for their full transcription (*16, 17*). This construct activates NF-AT–dependent transcription from the proper initiation site (*18*) in response to antigen presentation (*19*) or pharmacological agents such as phorbol myristate acetate (PMA) and the calcium ionophore ionomycin (*20, 21*). The concentration at which 50 percent inhibition of signaling is observed (the IC_{50}) for FK506 is about 0.1 nM, but the FK1012s had almost no mea-

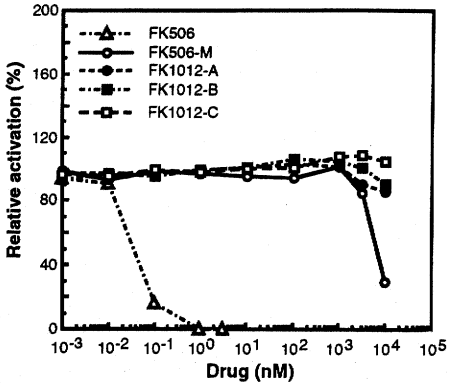

Fig. 2. Dimerization of FK506 eliminates its ability to block signal transduction. Jurkat-TAg cells (10^7) were subjected to electroporation with 4 μg of the SEAP reporter plasmid, NFAT-SX (*21*). After 24 hours subsets of these cells (~10^5) were stimulated for an additional 16 hours with ionomycin (1 μM) and PMA (25 ng/ml) plus 1:10 dilutions of the various allyl-derivatized FK506 analogs in 200 μl of growth media. After the cells were stimulated, 100 μl of heat-treated media (68°C, 1 hour) from each sample was examined for SEAP activity. The data are given as the activation (percent) relative to cells stimulated with ionomycin and PMA but without drug.

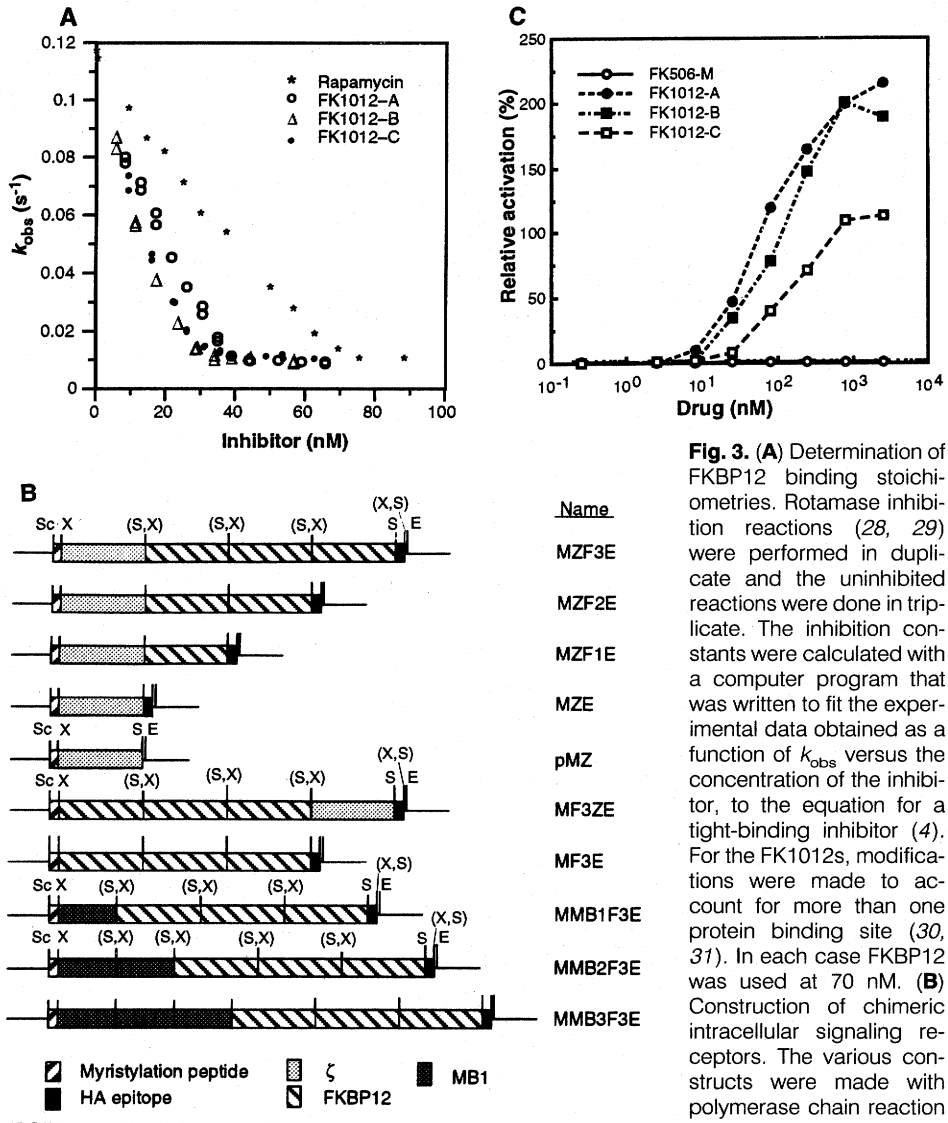

Fig. 3. (**A**) Determination of FKBP12 binding stoichiometries. Rotamase inhibition reactions (*28, 29*) were performed in duplicate and the uninhibited reactions were done in triplicate. The inhibition constants were calculated with a computer program that was written to fit the experimental data obtained as a function of k_{obs} versus the concentration of the inhibitor, to the equation for a tight-binding inhibitor (*4*). For the FK1012s, modifications were made to account for more than one protein binding site (*30, 31*). In each case FKBP12 was used at 70 nM. (**B**) Construction of chimeric intracellular signaling receptors. The various constructs were made with polymerase chain reaction (PCR)–amplified domains. The myristylation-targeting domain, residues 1 to 14 from c-Src (*38*), linked to the ζ cytoplasmic domain (*39*) was created with the use of 5′ primer 8908 and 3′ primer 8462 and the TCR ζ chain–containing plasmid TTZ (*36, 42*). The resulting (450 bp) Sac II–Eco RI fragment was cloned into the polylinker of the eukaryotic expression vector pBJ5 (in which the Sal I and Xho I sites have been destroyed), a derivative of pCDL-SRα (*43*), to give plasmid pMZ. The influenza HA epitope was created from hybridized oligonucleotides 8922 and 8923 and cloned into the Sal I site of pMZ to give MZE (*42*). The FKBP12 module (320 bp) was derived from hFKBP (*44*) with the primers 6052 and 6053 and cloned into the Xho I or Sal I site of MZE to give the various ζ-FKBP receptors (*42*). The 200-bp human MB1 module was derived from pGEX-MB1 (*45*) with the use of primers 10230 and 10231 and cloned into the Xho I site of MF3E to give MMB1F3E. The PCR constructs were verified by dideoxy sequencing, and full-length expression was verified by immunoblotting with mAb 12CA5 (BABCO). Sc, Sac II; X, Xho I; S, Sal I; and E, Eco RI. The (X,S) and (S,X) symbols represent the ligation of Xho I and Sal I half-sites, which destroys both sites. (**C**) Activation of signaling by FK1012s. Various dilutions of the FK1012s were added to samples of Jurkat-TAg cells cotransfected with 4 μg of NF-AT-SX and 5 μg of expression vector containing MZF3E. Transfections and SEAP assays were carried out as described in Fig. 2 (*21*), and the activations are displayed as a percentage of the activation incurred by mitogen stimulation (ionomycin at 1 μM and PMA at 12.5 ng/ml) on samples from the same transfections. The data represent the average from two wells and represent two independent experiments.

surable inhibitory effect on signaling at concentrations as high as 10 μM (Fig. 2). The derivatized monomer (FK506-M) was only slightly more inhibitory than the corresponding dimers at high ligand concentrations. As would be expected, none of the FK1012s had stimulatory activity toward the NF-AT reporter gene construct in the absence of mitogen (*22*).

Binding and stoichiometry of FK1012s to FKBP12. Several methods for determining both binding and inhibition constants between FKBP12 and its ligands have been developed (*4*). However, analyses of the data from these assays were based on the assumption that FKBP12 and its ligands form 1:1 complexes. Although the FK1012s were designed such that each FK506 molecule would bind to FKBP12 regardless of the other FK506 domain, there was no guarantee that steric effects would not interfere with the second binding event. Accordingly, we investigated the binding stoichiometries and affinities of the FK1012s by analyzing their ability to inhibit the enzymatic activity of FKBP12 and by accounting for their ability to bind two equivalents of FKBP12.

FKBP12 and other rotamases catalyze the interconversion of the cis- and trans-rotamers of peptidyl-prolyl amide bonds. The structures of FKBP12, both free (*23, 24*) and complexed with FK506 (*25*) and rapamycin (*26*), reveal the existence of a single ligand-binding site, which is also the site of rotamase catalysis. Thus, all known ligands of FKBP12 are inhibitors of its rotamase activity (*2, 27–31*).

To achieve maximum rotamase inhibition, the FK1012s required a preliminary incubation period (0 minutes ≤ incubation time ≤ 30 minutes) at 23°C, whereas neither FK506 nor rapamycin displayed such time-dependent inhibition. In the presence of 70 nM of FKBP12, the FK1012s behaved as two independent FK506 domains as judged by their ability to inhibit FKBP12 at only 35 nM FK1012 (Fig. 3A). In the case of FK1012-A, the two inhibition constants (K_i's) were determined to be 0.06 nM and 0.76 nM. For comparison, the K_i of rapamycin was found to be 0.16 nM. Thus, FK1012s are capable of binding two FKBP12 domains and both binding events occur with high affinity.

Signaling from the ζ chain of the T lymphocyte antigen receptor when crosslinked from within the cell. The signal transduction events initiated by contact with antigen are thought to be initially mediated by the tyrosine activation motif (TAM) (*3, 32*) that is repeated three times in the ζ chain of the TCR complex (*33, 34*) and singly in the γ, δ, and ε chains of CD3 in the complex. Chimeric proteins that consist of an extracellular ligand-binding domain, a transmembrane domain, and the cytoplasmic domain of ζ can initiate a signaling cascade, after their extracellular crosslinking by antibodies, that is indistinguishable from that initiated by crosslinking the TCR (*35–37*). We designed a chimeric signaling receptor that could be activated from within the cell by using cell permeable FK1012s to aggregate the receptor. The chimeric Src-ζ-FKBP12 receptor consisted of the NH_2-terminal 14 amino acids from c-Src (these residues allow myristylation and thus membrane association) (*38*) linked to the cytoplasmic domain of the ζ chain of the TCR, which in turn was fused to one to three tandem copies of FKBP12, the last of which contained the hemagglutinin (HA) epitope from influenza that is recognized by the monoclonal antibody (mAb) 12CA5 (*40–45*) (Fig. 3B). These constructs (MZF1E, MZF2E, and MZF3E) were cotransfected into Jurkat-TAg cells along with the SEAP reporter plasmid NF-AT-SX (*8*). As a positive control for activation, and as a reference for transfection efficiency, samples of transfected cells were stimulated with the mitogens ionomycin and PMA, and then assayed for SEAP activity. Samples of the same transfectants were titrated with FK1012s and their supernatants were assayed for SEAP activity. Each FK1012 was able to stimulate potently the MZF3E transfectant and to induce NF-AT as well as or better than the mitogens (Fig. 3C).

Multimerization of the FKBP12 domain had a synergistic effect on activation. The degree of activation seen with the tandem trimer was similar to that induced by ionomycin and PMA; the dimer, however, had only 5 to 10 percent of the activity of the trimer. The monomer, MZF1E, and a construct lacking an FKBP domain, MZE, were completely inactive (Fig. 4A). The addition of a fourth FKBP domain to MZF3E did not confer additional sensitivity to the crosslinking agent (*22*). This stimulation appeared to be dependent on aggregation, because the allyl-derivatized monomer FK506-M was completely inactive. Also, FK506-M could competitively inhibit the activity of FK1012-A when equimolar (or greater) amounts of the monomer were added to the activation reactions (Fig. 4B).

A comparison of the activation properties of three of the FK1012s suggested that the nature and length of their tethering element has little effect on their stimulatory constant (EC_{50}) values (Fig. 3C). However, when dimers were prepared with the same tethering elements and attached to the hydroxyl group on the cyclohexyl ring of FK506, a site not hindered by FKBP12 binding (*25*), little activation was observed. Binding studies with these dimers demonstrated that they do not readily form 1:2 complexes with FKBP12 (*46*). Finally, the activity of the NF-AT–responsive promoter could be finely regulated by several orders of magnitude by the use of FK1012 concentrations that also spanned several orders of magnitude.

Signaling through the TCR of mature peripheral T cells activates a specific set of transcription factors in the nucleus and

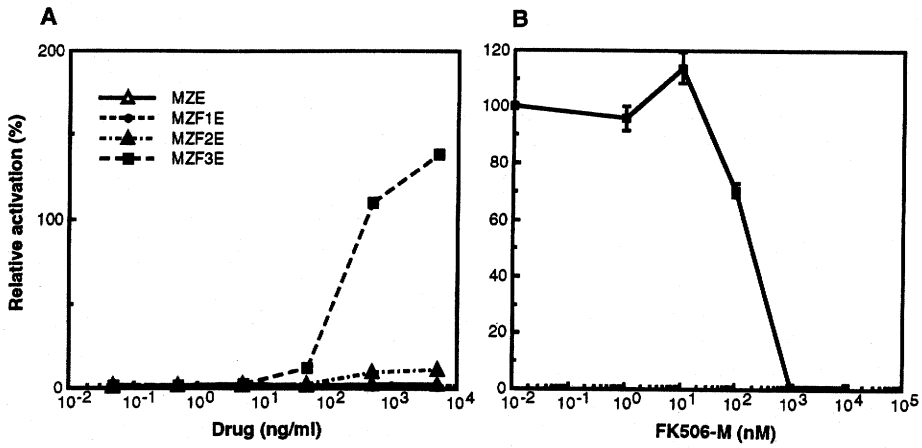

Fig. 4. (**A**) The effect of oligomerization of the FKBP12 domain on receptor signaling. Samples of FK1012-D were added to portions of Jurkat-TAg cells cotransfected with 4 μg of NF-AT-SX and 5 μg of expression vectors that contained various chimeras. Transfections and SEAP assays were performed as above, and induction of the reporter gene was measured as a percentage of the activation incurred by mitogen stimulation (ionomycin at 1 μM) and PMA at 25 ng/ml on samples from the same transfections. Each data point is the average from two samples and the results represent two independent experiments. (**B**) Activation of the myristoylated ζ-FKBP12 chimeras by crosslinking. Jurkat-TAg cells were cotransfected with 4 μg of NF-AT-SX and 5 μg of MZF3E. Log dilutions of monomeric FK506-M were added along with 100 nM of dimeric FK1012-A. Activation is displayed relative to that of 100 nM of FK1012-A alone. The data given are the average of two experiments, each containing two samples per dilution.

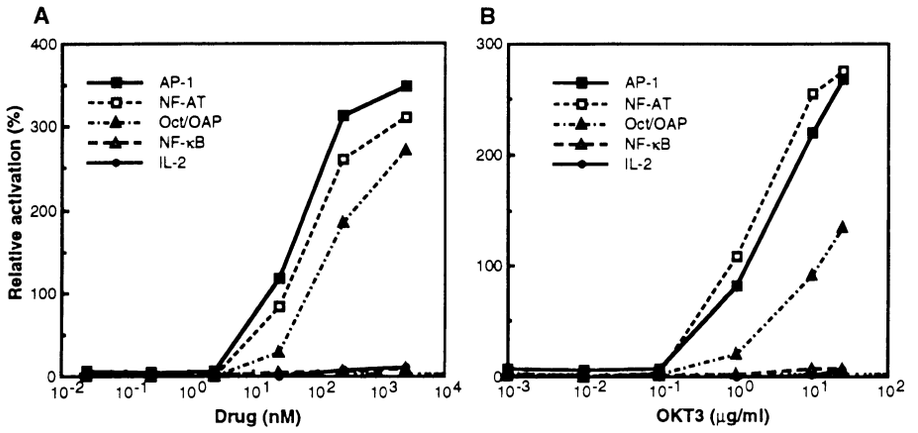

Fig. 5. Comparison of the induction of transcription factors by (**A**) FK1012-A or (**B**) OKT3 antibodies to the TCR. Jurkat-TAg cells were cotransfected with 4 μg of various SEAP reporter plasmids, in which five copies of the transcription element AP-1 [5′-TGACTCAGCGC-3′ from the human metallothionen promoter (*48*)], three copies of NF-κB [from κE2 in the murine κ chain enhancer (*49*)], three copies of NF-AT [nucleotides −286 to −257 (*18*)], or four copies of the antigen receptor response element (ARRE-1) that binds to the OAP–Oct-1 complex (*20*) binding sites directed expression of the SEAP reporter gene (*50*), and 5 μg of MZF3E as above. Each reporter plasmid contained a multimerized oligonucleotide containing the binding site for a distinct IL-2–enhancer-binding transcription factor within the context of a minimal IL-2 promoter (squares and triangles), or alternatively, the intact IL-2 enhancer-promoter (closed circles). Cells were stimulated by tenfold dilutions as indicated of (A) FK1012-A as before or by (B) mAb OKT3–coated microtiter wells. The activations are displayed as a percentage of the activation incurred by mitogen stimulation (ionomycin at 1 μM and PMA at 12.5 ng/ml) on samples from the same transfections. Each data point is the average of two wells and the results represent two independent experiments.

initiates the transcription of early activation genes such as that encoding IL-2 (*47*). The fidelity of signaling by intracellular crosslinking was tested by comparing the results obtained with FK1012A to those obtained by activating the TCR itself with crosslinking antibodies. Crosslinking either the ζ-FKBP12 chimera MZF3E (Fig. 5A) or the TCR (Fig. 5B) induced the activation of the NF-AT, Oct-1–OAP (octamer-associated protein), and AP-1 (activating protein–1) transcription factors comparably; however, neither reagent could induce significant NF-κB (nuclear factor of the κ enhancer in B cells) activity, or activate the intact IL-2 enhancer (*48–50*). Additional PMA restored both of these activities (*22*). These results indicated that signal transduction induced by intracellular crosslinking faithfully reflects that induced by extracellular crosslinking with antibodies.

To determine if myristylation is required for the activation of the chimeric receptor, we mutated the NH_2-terminal glycine from the c-Src–derived targeting peptide to an alanine and generated the MΔZF3E construct. This change blocks myristic acid transfer to Src while maintaining normal NH_2-terminal peptidase processing (*51*). This single amino acid change resulted in a

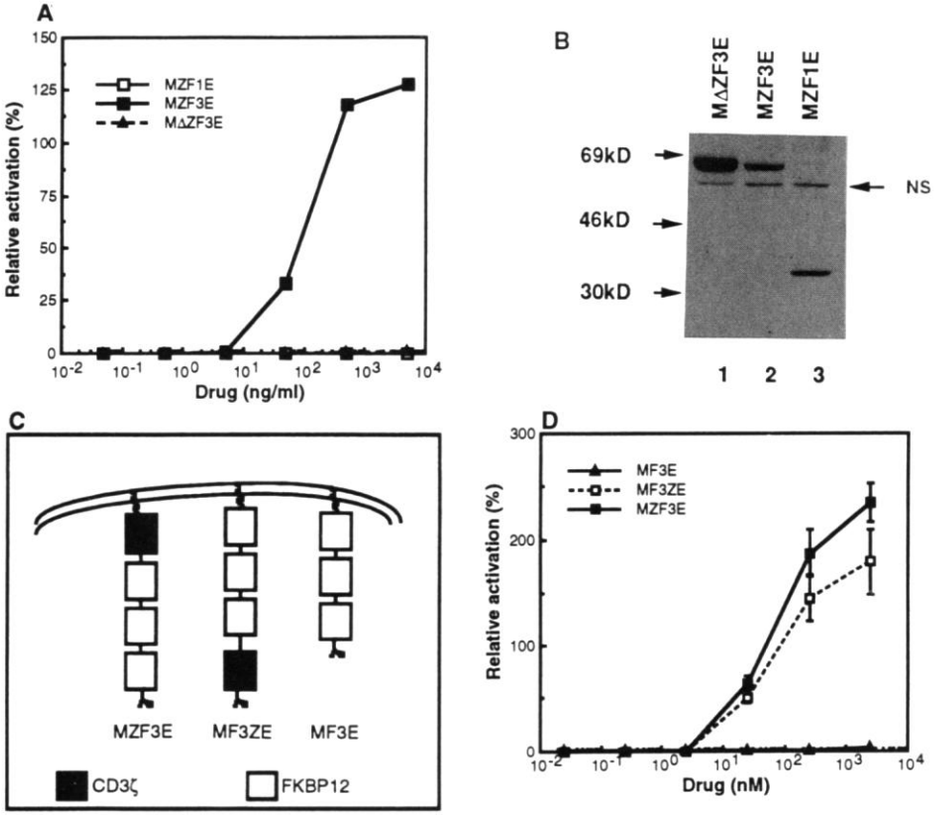

Fig. 6. (**A**) Membrane attachment is essential for the activation of the ζ-chain intracellular region. Jurkat-TAg cells were cotransfected with 4 μg of NF-AT-SX and 5 μg of MZF3E, the nonmyristoylated MΔZF3E, or control receptor, MZE, as above. The construct MΔZF3E was obtained with primers 9709 containing the Gly→Ala mutation and 8462 (*42*). The resulting PCR product was incubated with Sac II and Sac I and subcloned into Sac II–Sac I MZF3E that had been treated with Sac II and Sac I. (**A**) Transfections and SEAP assays were performed as above, and activations by FK1012-D are displayed as a percentage of the activation incurred by mitogen stimulation ionomycin at 1 μM and PMA at 25 ng/ml on samples from the same transfections. Each point is the average of two samples and the results represent two independent experiments. (**B**) Samples from the above transfections were lysed (0°C, for 30 min) in RIPA buffer [150 mM NaCl, 50 mM tris buffer (pH 8), 1 percent NP-40, 0.5 percent deoxycholate, and 0.1 percent SDS], diluted in 2× loading buffer (100 mM tris, 2 percent SDS, 280 mM β-mercaptoethanol, 20 percent glycerol, and bromphenol blue at 10 μg/ml) and subjected to electrophoreses on a 10 percent SDS-PAGE gel. Rainbow markers (Amersham) were used for molecular standards. After electrotransfer, blots were incubated first with mAb 12CA5 (BABCO) and second by horseradish peroxidase (HRP)–conjugated rabbit antiserum to mouse IgG (Zymed). Proteins were detected after a 10-sec exposure to x-ray film with ECL reagents (Amersham). NS, Nonspecific band. (**C**) and (**D**) Orientation-independence of FKBP12 and ζ domains in chimeric receptors. (C) Schematic of constructs used. (D) Transfections, activation with FK1012-A, and SEAP assays were performed as above. The activations are displayed as a percentage of the activation incurred by mitogen stimulation (ionomycin at 1 μM and PMA at 12.5 ng/ml) on samples from the same transfections. Each data point is the average of two independent experiments, each consisting of duplicate wells.

failure of the receptor to signal (Fig. 6A). The amount of the intracellular mutant receptor MΔZF3E, however, was higher than either MZF3E or the control receptor, MZF1E (Fig. 6B), suggesting that myristylation decreases steady-state protein concentrations, possibly by increasing the rate of protein degradation by an internalization mechanism.

In order to test whether the spacing of ζ relative to the plasma membrane was critical for triggering activation, we reversed the relative order of the ζ chain and the three FKBP12s. Because the activities of the two types of chimeras, MF3ZE and MZF3E, were similar (Fig. 6, C and D), the membrane-proximal positioning of the ζ cytoplasmic domain was not an absolute requirement for activation.

To determine whether FK1012s could be used to oligomerize and activate other receptors, we attempted to induce signaling through a component of the B cell antigen receptor complex. The single-TAM–containing proteins MB-1 and B29 are thought to function as B cell–specific analogs of the chains that comprise the CD3 and ζ components of the TCR (52). Therefore, the ζ moiety of MZF3E was replaced with the cytoplasmic domain of MB-1. This new chimeric receptor, MMB1F3E (Fig. 3B), was transiently transfected into Jurkat-TAg cells and assayed as above for FK1012-induced NF-AT activation (Fig. 7). Although the activation was 10 to 20 times less than that of the ζ-containing receptor MZF3E, it was significantly higher than the control receptor MF3E. The reduced activity may be because T cells are deficient in the protein tyrosine kinases, Syk and Lyn, found predominantly in B cells (53). Constructs containing two (MMB2F3E) or three (MMB3F3E) TAMs (Fig. 3B) were no more active than the single-TAM–containing MB-1 receptor, suggesting that the number of TAMs is not responsible for reducing the MB-1–chimera activity relative to the ζ chimera activity. In addition, immunoblots of extracts from transiently transfected cells with mAb 12CA5 showed that expression of MMB1F3E and MZF3E were similar (22), indicating that the amount of protein was not responsible for the differences.

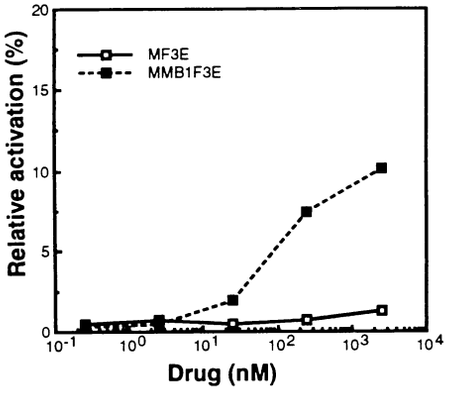

Fig. 7. The MB-1 intracellular domain can also be activated by intracellular crosslinking. Transfections and SEAP assays were performed as above, and the activations are displayed as a percentage of the activation incurred by mitogen stimulation (ionomycin at 1 μM and PMA at 12.5 ng/ml) on samples from the same transfections. Each data point is the average from two samples and the results represent two independent experiments.

Implications of FK1012-induced signaling. Signal transduction has been viewed largely through the experimental window of induction by extracellular ligands. Although many receptors and signaling molecules require membrane attachment for transformation or cellular activation, the reasons for this requirement are not well understood. One possibility is that cell surface receptors require a membrane-bound signaling domain for access to more distal molecules in the signaling cascade. Alternatively, intramembrane associations with other signaling molecules might be important for the function of membrane-bound receptors. Because all previously used ligands that crosslink cell surface receptors have acted on extracellular domains, discrimination between these explanations has been difficult. Our results indicate that, although intramembrane interactions are important for the assembly of the TCR complex (54), the ζ chain of the TCR and the MB-1 chain of the immunoglobulin receptor complex do not require their extracellular or transmembrane domains in order to initiate intracellular signaling. Despite the lack of a need for a transmembrane domain, intracellular proximity to the cell membrane appears to be essential for cell signaling from the ζ receptor element; removal of the myristyl group, which acts as a membrane anchor, resulted in a complete loss of activity. These findings indicate that for the two receptors examined, membrane proximity, but not a transmembrane region, is required for the activity of the receptor. Finally, the results do not indicate any necessity for a ligand-induced conformational change in ζ as an integral component of ζ-mediated signaling (35); structural investigations indicate that the binding

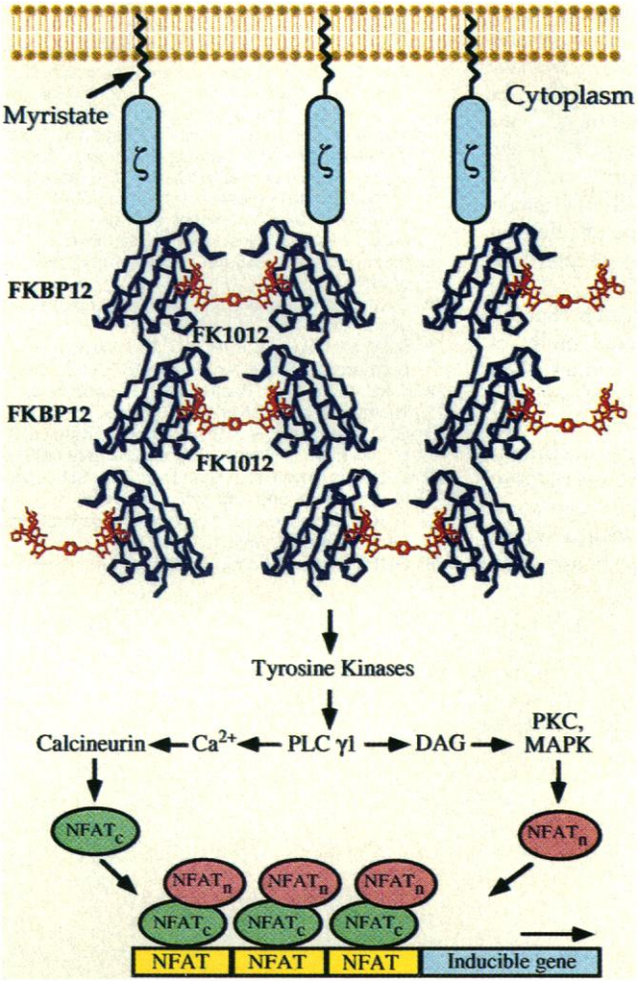

Fig. 8. Model for intracellular activation of MZF3E by FK1012s and subsequent signaling. Abbreviations are: PLCγ1, phospholipase Cγ1; DAG, diacyl glycerol; PKC, protein kinase C; MAPK, MAP kinase; and $NF\text{-}AT_{c,n}$, cytoplasmic and nuclear components of NF-AT, respectively.

of FK506 to FKBP12 induces few changes in the protein (*27*) and the ζ constructs of this study differ considerably from earlier constructs, yet are also competent for signaling.

The activation of signal transmission by FK1012s through the chimeric ζ-FKBP12 receptor requires concentrations of the crosslinkers that exceed, by approximately two orders of magnitude, the equilibrium binding constant of FK506 for FKBP12, K_d = 0.4 nM (*2*). This observation most likely reflects nonproductive interactions between the FK1012s and endogenous FKBPs. The synthesis of derivatives of FK1012s with substituents that interfere with binding to endogenous FKBPs and the construction of chimeric receptors with FKBP domains that have compensatory mutations should result in crosslinking at lower concentrations.

The intracellular expression of an artificial receptor that localizes to the inner leaflet of the plasma membrane has resulted in the regulated expression of a reporter gene (Fig. 8). Regulation is brought about by the ability of lipid permeable, synthetic ligands (FK1012s) to aggregate the receptor. We have taken advantage of the recent molecular dissection of the TCR-mediated signaling pathway (*10*, *55*) to create a ligand-induced signaling pathway that comprises, in part, a component of the TCR, yet does not activate the endogenous IL-2 gene. By cotransfecting reporter and receptor genes, we have synthesized a signaling pathway that can be activated with nontoxic, cell membrane–permeable FK1012s. This same pathway can be inactivated by the competitive removal of the crosslinking FK1012s with monomers such as FK506-M, or by the use of calcineurin inhibitors such as the immunosuppressive drugs cyclosporin A or FK506.

Because the dimerizing agents that we prepared could be administered orally or absorbed through skin or membranes, their ability to elicit the expression of specified genes in vivo may prove feasible. The most immediate application of this technique may be in the creation of transgenic animals with inducible phenotypes. Our study not only demonstrates how such a system might be developed, but it also points to an even more general capability of regulated homodimerization and heterodimerization of intracellular proteins in living cells.

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